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Effects of Glutamine in Neonatal Endotoxaemia

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Abstract

Background: Neonatal sepsis increases systemic inflammation, which may lead to multiple organ failure and death. Glutamine has been suggested to have beneficial effects during neonatal sepsis, but its effects on systemic inflammation and systemic metabolism are unknown.

Methods: Suckling rat pups were given intraperitoneal endotoxin alone (E) or with glutamine (EG). Controls were administered saline (C) alone or with glutamine (CG). Sepsis score and rectal temperature were monitored. Oxygen consumption (VO2, ml/kg/h) was measured by indirect calorimetry. Animals were sacrificed exsanguinated and plasma separated. Liver was resected and hepatocytes and liver mitochondria were isolated for *in vitro* experiments. Oxygen consumption and glutathione levels in hepatocytes and hepatocyte mitochondria were measured. Plasma glutamine and tumour necrosis factor alpha (TNF-α, pg/ml) were measured.

Results: Endotoxic (E) rats had lower VO2 than C rats from 90 minutes post injection to 210 minutes (VO2 150-210 minutes: C 671+/-45; E 429+/-36, $p<0.0004$; n=8). VO2 was higher ($p<0.05$) in EG rats than E rats (E 460+/-29; EG 654+/-68; n=10). EG rats were significantly less hypothermic between 90-210 minutes (58/132 measurements) compared with E (95/147; p=0.0007). Sepsis score was significantly lower in the EG group than the E group (E 4.9+/-0.3; EG 3.6+/-0.3; n=40; $p<0.01$).

Endotoxaemia caused a rapid significant decrease in plasma glutamine concentration at 2h (C *vs.* E $p<0.001$), which was prevented by intraperitoneal glutamine (EG $p<0.05$ *vs.* E). TNF-α levels were greatly increased by endotoxaemia and this increase was partly prevented by glutamine (At 6h C 32+/-27, E 799+/-193, EG 219+/-75, E *vs.* C $p<0.001$, *vs.* EG $p<0.01$).

In isolated hepatocytes there was reduced oxygen consumption in endotoxin (E) group but increased oxygen consumption in endotoxin glutamine (EG) group. There was shown to be no statistical difference between all groups in the hepatocyte isolated mitochondrial oxygen consumption. Glutathione levels in the liver were reduced in both E and EG groups compared to controls. Glutathione in liver mitochondria showed an increase in the groups treated with glutamine at 4 hours post injection.

Conclusions: Neonatal endotoxaemia lowers VO2, heat production, and body temperature. Glutamine restores VO2 of endotoxic animals. Glutamine additionally increases rectal temperature and improves clinical signs of endotoxic rats. Glutamine administration prevents the endotoxin-induced fall in plasma glutamine levels, and reduces the concentration of both pro- and anti-inflammatory cytokines, supporting its use as a potential therapeutic agent in neonatal sepsis.

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1 General Introduction

1.1 Introduction

Sepsis is an important cause of morbidity and mortality in neonates. Between 1-5% of live births have an episode of sepsis in the neonatal period and of these there is a mortality rate of 10-15%[1,2]. Those at particular risk include neonates whom are either premature or low birth weight, or needing mechanical ventilation or total parenteral nutrition[3,4], or those who have congenital gastrointestinal anomalies[1]. Suitable therapies and support for these patients are needed to prevent initial sepsis leading to organ failure and the subsequent cascade of multi-organ failure.

1.2 Sepsis

1.2.1 Neonatal Sepsis

In neonates, sepsis associated with central venous access lines is the most common cause, with coagulase-negative staphylococci the most common infectious agent[5]. However, sepsis related to the gastro-intestinal system with bacterial translocation or gut abnormalities allowing ingress of bacteria from the intestine is also of significant

incidence. Surgery on the gut or abnormalities of the gut, e.g. atresias, can increase the risk of bacteria entering the circulation.

Sepsis is such a serious problem in the neonatal period because of the relative immunological immaturity, especially in premature and low birth weight babies. Sepsis can therefore lead rapidly from bacteraemia to septicaemia and septic inflammatory response syndrome (SIRS) and then on to multi-organ failure. Sepsis has also been implicated in the pathophysiology of necrotising enterocolitis (NEC). Premature and low birth weight babies are also at risk of necrotising enterocolitis (NEC), which can lead to severe morbidity and mortality.

Necrotising enterocolitis (NEC) is a disease of the neonatal period in which parts of the bowel become gangrenous. The cause of NEC is not known, and the pathophysiology is poorly understood. A combination of ischaemia-reperfusion and inflammatory or septic events are suggested mechanisms leading to NEC. Rates of NEC in neonatal intensive care units have been reported up to 7.7% of all admissions. However, though overall infant mortality rates have improved with better neonatal intensive care, the mortality rate of NEC has increased from 11.5 to 12.3 per 100,000.[6] Patients with necrotising enterocolitis (NEC) have a high incidence of organ failure other than the gastro-intestinal system. More than 90% of infants with NEC have signs of respiratory failure, renal failure in 85%, cardiovascular failure in 33% and hepatic failure in 15%[7]. The number of systems involved correlates with the severity and the outcome of the disease. The more systems with signs of failure, the more severe the disease and the worse outcome with the highest morbidity and mortality rates[7].

1.2.2 Bacterial Translocation

Bacterial translocation is a term used to describe the passage of bacteria from the intestinal lumen across the mucosal barrier to mesenteric lymph nodes from where the bacteria can move in to the blood stream leading to bacteraemia and even septicaemia. Bacterial translocation is more likely under certain conditions. These can be split in to luminal and mucosal factors.

Conditions in the lumen of the gut that lead to bacterial overgrowth, e.g. stasis and intestinal dilatation are associated with an increased rate of bacterial translocation[8]. The nature of the bacteria colonizing the intestine can also lead to changes in the rate of bacterial translocation[9]. Certain bacteria, e.g. *Lactobacillus*, appear to be less invasive of the mucosal barrier, while treatment with antibiotics can denude the gut of its normal bacterial flora, thus allowing more virulent bacteria to flourish, which are more able to translocate across the mucosal barrier. Breast milk has been shown to have a beneficial effect, leading to reduced bacterial overgrowth and reduced bacterial translocation in the neonatal period[10]. Initial neonatal intestinal bacterial colonization occurs from the mother's intestinal flora at and after delivery. Breast milk contains large amounts of secretory IgA antibodies that will be directed mainly towards mother's recent gut microflora[11]. Breast milk therefore may be beneficial in preventing sepsis by reducing the neonatal gut bacterial growth in the early stages of life. There are also IgG antibodies in breast milk and these can be absorbed in to the body to assist in preventing infections from bacteraemias.

Mucosal factors leading to bacterial translocation include any condition that can lead to damage to the mucosa and resulting in increased permeability. Surgery on the gut is a risk factor for bacterial translocation. In order to limit the risk, the surgeon should always keep handling of the bowel to a minimum. Other diseases with damage to the mucosa, including necrotising enterocolitis, pancreatitis and colitis are also associated with increased bacterial translocation[8,12].

From previous studies there is evidence that Gram-negative bacteria are more likely to translocate and cause septic episodes than anaerobes or other gut flora[13]. There is debate over the significance of bacterial translocation in the aetiology of sepsis as there is less evidence in humans than in animal studies[13]. However, in parenterally fed neonates, there is evidence that clinically significant septic episodes are related to bacterial translocation in 86% of episodes in 76% of patients[3,13]. Parenterally fed patients are at higher risk of septic episodes, which is often related to gut bacterial translocation. Possible contributing pathways include an overgrowth of bacteria in the unused gut as well as decreased nutrients to the enteral mucosa leading to a reduced effectiveness of the protective function of the mucosal barrier. Long-term total parenteral nutrition can result in mucosal atrophy.

1.2.3 Metabolism in Sepsis

During sepsis and septic events there are many factors affecting metabolism. Studies in adults have suggested that there are two phases in the metabolic response to sepsis, systemic inflammatory response syndrome, surgery or trauma: a

hypometabolic (ebb) phase, followed by a more prolonged hypermetabolic (flow) phase[14,15,16]. Although infants and children with sepsis or systemic inflammatory response syndrome frequently present with hypothermia[17], it is not known whether there is a short-lived hypometabolism which may contribute to the hypothermia observed in these patients. Studies in infants and children have suggested that the hypermetabolic phase may not occur[18,19,20,21] or may be short-lived following surgery[22]. Turi et al showed that there was no change in overall resting energy expenditure (REE) in children with septic inflammatory response syndrome (SIRS) or sepsis. It has been speculated that children may be diverting the energy being used for growth into the recovery processes, and that, therefore, there may be no overall change in metabolism, as seen in Turi et al[21], but instead a shift in the type of metabolism occurring. This would require that there was a reduction in metabolism used for growth at the time of SIRS or sepsis, at the same time as the corresponding hypermetabolic effect of SIRS or sepsis. Thus overall no change in metabolism would be measured. It is also speculated that there is a diversion of protein synthesis from growth to tissue repair. Similar results were seen in critically ill premature neonates who showed no increase in resting energy expenditure and no increase in energy expenditure on the first day post surgery[23].

In animal studies on rats there is hypo-metabolism[24] in the early stages of endotoxin administration, which is then followed by a hyper-metabolic phase. There was therefore hypothermia in the early phase of administration of endotoxin in rats followed by hyperthermia. Derijk et al showed that the hypothermic phase was attenuated in rats with eliminated peripheral macrophages[24]. Derijk et al also showed

that the hypothermic phase was also attenuated by rats treated with a biologically active antiserum to tumour necrosis factor alpha (TNFα)[25]. They therefore concluded that peripheral macrophage release of TNFα is involved in the hypothermic response to endotoxin seen in rats. The hyperthermic phase following the hypothermic phase in rats is also attenuated by elimination of peripheral macrophages and it is suggested that cytokine release from the macrophages is involved in the pyrogenic and thermogenic response of rats to endotoxin (LPS)[26].

1.2.4 Substrate metabolism in sepsis

Studies in adults have shown that the substrates used by the body during sepsis can vary depending on the available nutrients. Carbohydrates and particularly glucose is the main fuel, particularly for the brain, under normal physiological conditions. When there is a deficiency in carbohydrate supply and glycogen stores decrease then fat and protein are used to provide a higher proportion of energy[15]. In conditions of sepsis and starvation there is therefore an increase in lipolysis as carbohydrate and particularly glycogen stores become depleted as well as increased catabolism of muscle to generate amino acids, which can also be used as an energy substrate. The contributions of carbohydrate, fat and protein to energy production under conditions of sepsis depends on the enteral or parenteral supply of nutrition as well as on the energy requirements, which can in sepsis be increased.

1.3 Metabolism of Glutamine

Glutamine is the most abundant amino acid in the circulation. It is a five-carbon amino acid with two amino groups on it. Glutamine accounts for a third of all the amino acid nitrogen that is transported in the plasma. It has many uses in the body. Glutamine under normal metabolic conditions is a non-essential amino acid. i.e. the body's glutamine requirement can be synthesized from other substrates and there is no requirement for dietary glutamine. Pyruvate, which is formed in glycolysis of glucose, enters the citric acid cycle and is then converted to α-ketoglutarate, which can then be converted to glutamate by glutamate dehydrogenase with the incorporation of ammonia (Figure 1). Glutamate can also be formed by transamination of other amino acids. Glutamate can then be converted to glutamine by the addition of a further amino group by glutamine synthetase, utilizing ammonia as the amine donor and ATP as the energy substrate (Figure 2). Thus under normal metabolic conditions an intake of glutamine is not required as the body's glutamine requirement for metabolism and protein synthesis can be synthesized instead. In addition formation of glutamine is an important route for detoxification of ammonia.

Figure 1 **Glutamate synthesis from α-ketoglutarate**

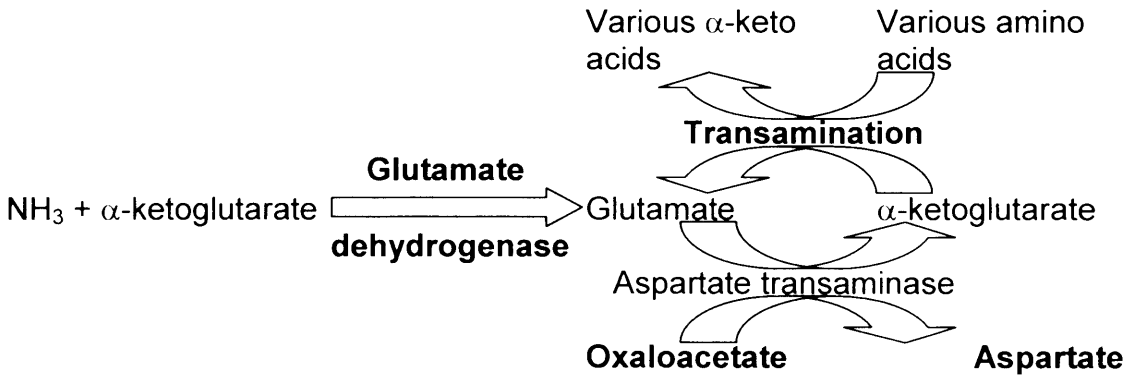
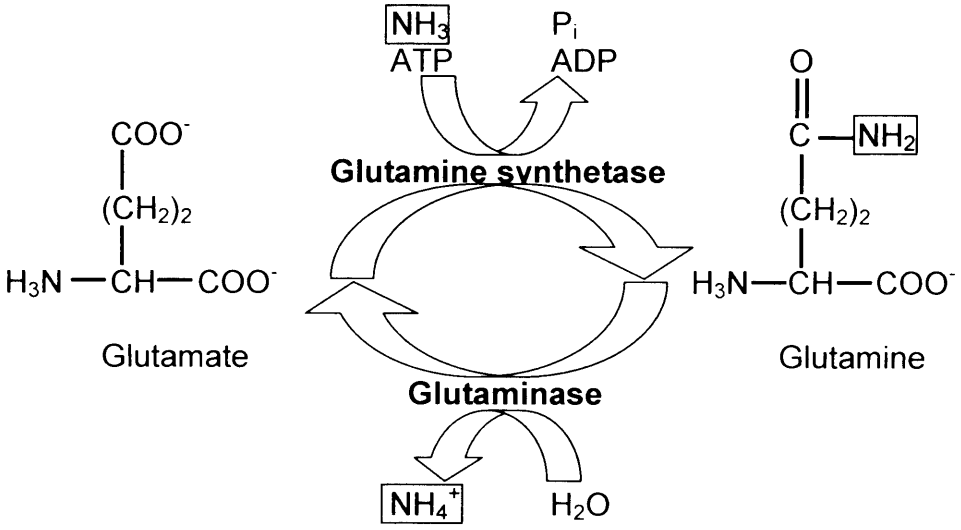


Figure 2 **Glutamine synthesis and degradation**



1.3.1 Fuel source

Oxidation of glutamine can be used to generate adenosine triphosphate (ATP). At the inner mitochondrial membrane the enzyme glutaminase converts glutamine to glutamate and ammonia. The glutamate is then converted via glutamate dehydrogenase to α-ketoglutarate and ammonia, which diffuses out of the cell. The α-ketoglutarate then enters the citric acid cycle to be converted to carbon dioxide and water while releasing energy in the form of ATP. Glutamine is also used via glutamate for the transamination of pyruvate to alanine by alanine amino transferase (ALT), which also produces ATP. In some cells e.g. enterocytes and lymphocytes glutamine is a primary energy source. However, under physiological conditions there is an increase in glutamine oxidation as glucose levels drop[27] and thus glutamine can play an important role when glucose levels are low. Cells can therefore use glutamine as an alternative fuel source.

1.3.2 Nitrogen transport

Glutamine is the main transport carrier of nitrogen in the body with one third of nitrogen carried in this way[28]. Glutamine is used to transport nitrogen via the blood as a non-toxic way of carrying ammonia from the peripheral cells to the liver and kidneys. At the liver or kidneys glutamine can be hydrolyzed to glutamate by the enzyme glutaminase releasing ammonia which in the liver can then be used in ureagenesis. Glutamine is the main source of nitrogen used in hepatic ureagenesis[29].

Glutamate can also be further hydrolyzed to α-ketoglutarate by glutamate dehydrogenase generating ammonia also. Ammonia can combine with carbon dioxide forming carbamoyl phosphate, which is a substrate for ureagenesis.

In the liver there is an uneven distribution of enzymes, involved in glutamine metabolism across the hepatic sinusoid[30]. In periportal cells, there is a net consumption of glutamine as there is a high concentration of glutaminase. The ammonia thus generated then becomes a substrate for ureagenesis and thereby is processed for excretion via the kidneys. In perivenous cells, glutamate is combined with ammonia and glutamine synthetase to produce glutamine, which is then transported around the body via the blood. The balance between the hydrolysis of glutamine at the periportal hepatocytes and the synthesis of glutamine at the perivenous hepatocytes thus controls the net transport of ammonia from the peripheral tissues to the liver and the transport back of glutamine from the liver to the peripheral tissues, and net excretion as urea.

1.3.3 Production of Amino acids and protein synthesis

Glutamate produced from glutamine by glutaminase can be used as an amine donor to help synthesize other amino acids. For example, the transamination of glutamate with pyruvate to produce alanine is catalyzed by alanine aminotransferase[31]. As well as producing alanine this also produces α-ketoglutarate that can be oxidised in the citric acid cycle to give ATP as an energy source for further reactions in the cell.

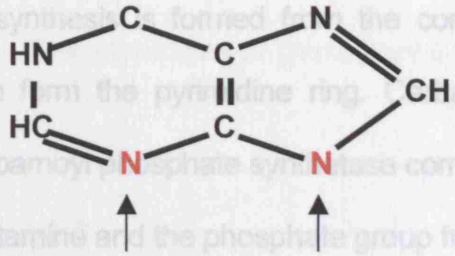
As well as glutamine being used to help synthesize other amino acids including glutamate and alanine, glutamine is also incorporated in to protein production directly as an amino acid and is required in the de-novo synthesis of enzymes and other proteins.

1.3.4 Nucleotide precursor

Glutamine is essential for the endogenous synthesis of purines and pyrimidines. Purine and Pyrimidine bases are poorly absorbed by the intestine and the absorbed bases are not delivered to the tissues as they are degraded in the intestinal mucosa. Therefore we depend on endogenous synthesis of purine and pyrimidine bases to be used in the production of ribonucleotides and deoxyribonucleotides. Ribonucleotides e.g. ATP, GTP, CTP, UTP are important coenzymes in metabolism. Deoxyribonucleotides are required for DNA replication and repair.

The liver is the main site of synthesis of purines. The bases and nucleosides are then exported to other tissues. Purines are synthesised from ribose-5-phosphate, from the pentose phosphate pathway. PRPP synthetase enzyme transfers a pyrophosphate group from ATP on to ribose-5-phosphate forming 5-phophoribosyl-1-phosphate (PRPP). Glutamine then acts as an nitrogen donor transferring an amide group to PRPP. Following this the purine ring is constructed from simple precursors with glutamine and aspartate donating further nitrogen groups. Glutamine therefore supplies two nitrogen groups (Figure 3A).

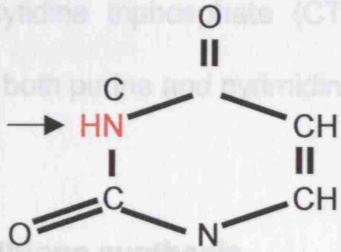
Figure 3 Glutamine incorporation into nucleotides



Nitrogen atoms donated by glutamine

A. Purine Ring

Glutamine is the source of two of the nitrogen atoms in purine ring formation



B. Pyrimidine Ring

Glutamine is the source of one of the nitrogen atoms in the pyrimidine ring formation

Drugs that interfere with glutamine utilisation are inhibitory to purine synthesis e.g. azaserine (glutamine antagonist).

Pyrimidine synthesis is formed from the combining of carbamoyl phosphate with aspartate to form the pyrimidine ring. Carbamoyl phosphate is produced by the enzyme carbamoyl phosphate synthetase combining carbon dioxide with the nitrogen group of glutamine and the phosphate group from ATP (Figure 3B).

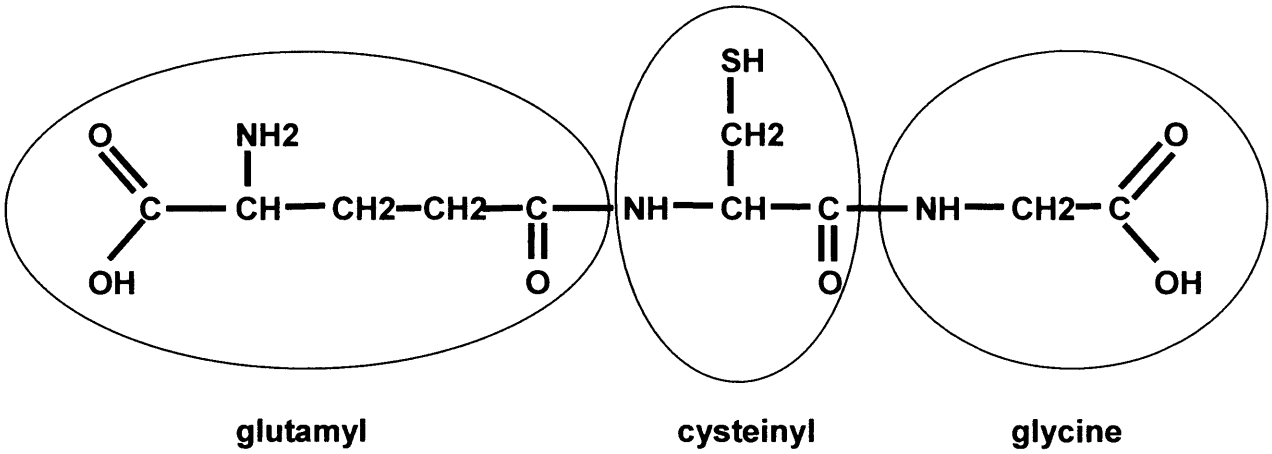
Glutamine is also used to convert the purine, inosine monophosphate (IMP) into guanosine monophosphate (GMP) as well as the pyrimidine, uridine triphosphate (UTP) into cytidine triphosphate (CTP). Glutamine is therefore essential to the production of both purine and pyrimidine bases[32].

1.3.5 Glutathione synthesis

Glutamine plays an important role in cellular redox control[33]. It does this by regulating glutathione synthesis. Glutathione (GSH) is an antioxidant that protects against reactive oxygen species that cause oxidative cellular damage.[34] Glutamine can be converted to glutamate, which is one of the constituents of glutathione. Glutamate, cysteine and glycine are combined to form glutathione (Figure 4). Intracellular glutamate, derived from glutamine, is exchanged across the cell membrane for cysteine. Therefore the requirement of glutamate concentration inside the cell for the production of glutathione must allow for the amount incorporated in the glutathione as well as the concentration of glutamate needed for exchange with cysteine. Some cell types, e.g. erythrocytes, do not possess a cell-membrane glutamate transporter and

so they have an absolute requirement for glutamine to provide glutamate for glutathione synthesis.

Figure 4 The structure of glutathione (γ-glutamylcysteinyl glycine)



Glutathione also has an important function as an antioxidant[35]. All aerobic organisms are subject to a certain level of physiological oxidative stress with the production of hydrogen peroxide and oxygen radicals, such as superoxide (O_2·) which can cause cell injury. Under stress conditions e.g. sepsis there can be an increase in toxic metabolites produced. Glutathione (GSH) reacts with hydrogen peroxide or with reactive oxygen species in the presence of a GSH peroxidase to produce oxidised glutathione (GSSG). GSSG can then be converted back to GSH by GSH reductase at the expense of NADPH. However if there is severe oxidative stress, e.g. sepsis, this may overcome the ability of the cell to reduce GSSG to GSH leading to accumulation of GSSG within the cell. To protect the cell from a build up of GSSG and the subsequent shift in redox equilibrium, GSSG can either be actively exported from the cell or can react with a protein sulfhydryl group to form a mixed disulfide.

Glutathione is thus essential for maintaining the intracellular redox status[36]. Glutathione can maintain redox in cells by reacting with protein thiol groups in a reversible reaction that equilibrates according to the redox state of the cell and is catalyzed by thiol-transferase:

Protein-SSG + GSH ⟶ protein-SH + GSSG

This thiol-disulfide equilibrium in cells is also known to regulate diverse enzyme activity, transport activity and gene expression.

1.3.6 Glutamine and glutamate in response to metabolic acidosis

In response to reduced body fluid alkaline reserves proximal tubules of the kidney increase glutamine uptake. There is then an increase in the flux of glutamine being converted to glutamate and ammonium (NH_4^+) by glutaminase, at the inner mitochondrial membrane. The glutamate thus produced then enters the glutamate dehydrogenase pathway in the mitochondria that converts glutamate to α-ketoglutarate and a second ammonium (NH_4^+) ion. The α-ketoglutarate is then broken down in the citric acid cycle giving adenosine triphosphate (ATP). With complete oxidation of α-ketoglutarate 2 molecules of bicarbonate (HCO_3^-) are produced. The ammonium is then excreted in to the urine while the bicarbonate is transported out of the cell into the renal vein. This thus gives a net gain of two molecules of base that can then balance against the metabolic acidosis[31].

1.3.7 Glutamine requirements and effects on immune cells in vitro

In 1949 glutamine was shown to be an essential amino acid for *in vitro* cell growth in tissue culture[37]. The proliferative response of both rat and human lymphocyte cells has been shown to be dependant on glutamine[38,39]. Proliferation of rat lymphocytes has been shown to increase four-fold when glutamine was added to the medium. This effect was not seen with the substitution of other amino acids for the glutamine[40].

Glutamine also can affect the activity of immune cells as well as their proliferation and growth. The rate of phagocytosis in mouse macrophages has been shown to decrease as glutamine concentrations drop in a dose-response curve[41]. This has been shown to occur in the physiological range of glutamine concentrations.

Other work looking at human monocyte derived antigen expression, showed a decrease in MHC Class II antigen of 40% when associated with a decrease in glutamine concentration from 2mmol/l to 200μmol/l. There was also a decrease in both phagocytosis and opsonization of *Escherichia coli*[42].

Neutrophils harvested from children who had sustained major burns have been shown to have a deficit in neutrophil bactericidal function. This deficit is improved by addition of glutamine[43]. The neutrophils also showed improved ability to kill *Staphylococcus aureus* with glutamine, and in some cases the neutrophil function was restored to normal or even greater than normal levels. Neutrophils harvested from post-operative patients also showed an improvement in bactericidal function when incubated with increasing concentrations of glutamine from 100μmol/l up to

1mmol/l[44]. Glutamine, in suitable concentration, is therefore essential for the normal differentiation and proliferation of immune cells.

1.4 Glutamine turnover

Glutamine has traditionally not been used in parenteral nutrition due to its poor solubility and stability[45]. Therefore experiments to assess the effect of administration of glutamine under these conditions of catabolic stress are needed to assess the effect of glutamine both in physiological doses to maintain physiological glutamine levels and also in pharmacological doses.

To understand the theories behind the administration of glutamine in stress related situations e.g. trauma, surgery or sepsis, it is necessary to have an understanding of the metabolism of glutamine and its usage by cells in the body.

1.4.1 Glutamine deficiency — does it happen?

The enzyme glutamine synthetase is present in many different cell types in the body, and these cells maintain the ability to synthesize glutamine. Most organs do not act as net producers of glutamine. The lung maybe a net producer of glutamine under acute conditions[46], but the main net producer of glutamine is skeletal muscle, due to its large mass[28]. Muscle glutamine synthetase is upregulated in acute stress response situations and therefore results in large export of glutamine from the skeletal muscle and skeletal muscle also produces glutamine by breakdown of

protein. The initial release of glutamine from skeletal muscle at the onset of stress conditions causes the intracellular glutamine concentration to lower. This lowering of intracellular glutamine concentration leads to increased protein breakdown and glutamine synthetase production of glutamine from other amino acids. Glutamine is transported from skeletal muscle via the blood and is specifically taken up by the liver, kidneys, lymphatics and bowel[47].

Glutamine under normal conditions is a non-essential amino acid that can be synthesized in sufficient quantities to supply all requirements. There is therefore no requirement to ingest glutamine under standard conditions. In times of catabolic stress e.g. sepsis, trauma, surgery there can be depletion of glutamine, leading to lower plasma glutamine levels and in particular intracellular glutamine depletion[48]. In the late 1980s and early 1990s suggestions were made that glutamine may be conditionally essential under certain catabolic conditions. These conditions included during sepsis, surgery, trauma and cytotoxic therapy. Animal studies showed that a laparotomy could lead to a drop of between 30-50% in plasma glutamine concentrations[49]. In patients undergoing vascular operations a 50% drop in plasma glutamine concentrations has been noted[50]. During these catabolic states there was noted to be an increased turnover of glutamine with increased production of glutamine by the liver and muscles and increased usage of glutamine particularly by the intestine and immune cells[51,52].

Under conditions of stress, e.g. surgery or sepsis, the metabolism of glutamine is increased and this is when glutamine may become conditionally essential, i.e. the metabolism and consumption of glutamine may outstrip the production of glutamine

and therefore glutamine ingestion may be required to maintain body glutamine levels[53]. In the inflammatory response glutamine is consumed in increased amounts by immune cells both in the circulation and in the tissues[54]. These additional requirements for glutamine by immune tissue and the increased utilization for the other metabolic pathways mentioned above means that utilization of glutamine could outstrip the local production of glutamine, resulting in a fall in plasma glutamine. This in turn triggers increased production of glutamine, particularly in skeletal muscle to counteract the drop in glutamine levels[55].

In addition to the drop in glutamine concentration in plasma, cells which are further from the capillaries may be exposed to even lower glutamine concentrations as the cells between them and the capillary extract glutamine and thus lower the concentration of glutamine in the extracellular fluid below that in the plasma. In the liver, cells around the portal sinus are likely to be exposed to lower concentrations of glutamine, as the portal venous blood has already had glutamine extracted by the small bowel and thus has a lower glutamine concentration than arterial blood. Therefore it is reasonable to suppose that immune tissue in the portal sinus is compromised because of decreased glutamine concentrations[54] both in the liver and in the gut.

In severe or prolonged injury, despite the increase in mobilization of glutamine from the muscles, glutamine pools are rapidly depleted[56]. Plasma glutamine levels and muscle glutamine levels have been shown to fall though whether total body glutamine falls to sufficiently low levels to become a deficiency of glutamine is debatable[57]. With the abundance of glutamine seen in the plasma and the body as a

whole, a significant stimulus to consumption would have to occur in order for a truly whole body deficient state to be reached. While it is uncertain if whole body glutamine deficiency occurs, local decreases in glutamine, particularly intracellular glutamine, have been measured[58].

1.5 Studies of glutamine administration

1.5.1 Adult human studies

Glutamine has been administered in catabolic stress conditions to adults with variable results. There has been shown to be a decrease in mortality in intensive care patients shown at six months post admission to the Intensive Care Unit (ICU)[59]. However it is hard to explain how a glutamine supplemented nutrition at the time of intensive care admission led to a decrease in mortality at six months though not to a statistical difference in early mortality. However, this could be due to a lack of power in the study to show early effects. The difference in six month mortality was however almost completely described by those receiving 5 or more days glutamine treatment; 9 of 25 versus 18 of 27 using the control nutrition ($p<0.05$). There was shown to be a decrease in catheter related infections in the glutamine group ($p=0.026$). Of the mortality from multi-organ failure in the intensive care unit, 8 of 8 in the glutamine group and 14 of 16 in the control group sustained one or more Intensive care acquired infections and accounted for 38% versus 74%, respectively, of the acquired infections occurring in those patients[60].

Also *candida* sepsis was reduced in the glutamine group and there was zero mortality from *candida* infection despite there being a similar incidence of colonisation. This was in contrast with the control group where there was a higher incidence of *candida* infections as well as 6 patients with *candida* infection who died of multi-organ failure ($p=0.02$). In conjunction with an improvement in clinical data there was also a reduction in cost of intensive care stay as well as cost per survivor[61].

Further clinical studies have shown an improvement in outcome of multi-trauma patients. Houdijk et al showed that, with nasoduodenal enteral feeding of glutamine supplemented feeds compared to control feeds, there was a decrease in pneumonia ($p<0.02$), a decrease in bacteraemia ($p<0.005$) and a decrease in sepsis ($p<0.05$). There was however no reduction shown in either intensive care or hospital stay nor in the number of days on a ventilator[62].

In multiple trauma patients Long et al had previously shown that there was a reduction in plasma glutamine levels seen following multiple trauma of up to 50%[63]. On this and other similar observations the hypothesis for glutamine supplementation was based. Kudsk et al[64] showed that an immune enhancing diet, including glutamine supplementation, significantly reduced major infectious complications in severely injured patients compared with those who received an isonitrogenous diet or no early enteral nutrition. They concluded that an immune enhancing diet was the preferred diet for early enteral feeding after severe blunt and penetrating trauma as these patients are at risk of subsequent septic complications. Unfed patients had the highest complication rate.

Kudsk et al had also previously shown that enteral feeding in multiple trauma patients had a beneficial reduction in the number of patients becoming infected and with the number of infectious episodes per patient compared to parenterally fed multiple trauma patients[65]. However studies in parenterally administered glutamine supplemented feeds have shown beneficial reductions in infectious episodes under catabolic stress situations. Wischmeyer et al[66] showed that glutamine supplemented parenteral nutrition in burns patients reduced the number of bacteraemic episodes due to gram negative organisms as well as improving measures of nutrition and decreasing measures of inflammation. There was also a trend towards a reduced total number of bacteraemic episodes, a reduced mortality and a decreased usage of antibiotics in glutamine supplemented group compared to an isonitrogenous parenterally fed control group.

Further studies post major abdominal surgery have also shown a trend of evidence towards the beneficial effects of glutamine supplementation[67,68,69]. A reduction in plasma glutamine levels post-operatively has been noted with plasma glutamine levels remaining low for up to 7 days post-op. The beneficial effects of glutamine supplementation in major abdominal surgery could be due to the maintenance of plasma glutamine levels in the glutamine supplemented groups compared to controls[67].

However conclusive evidence of the benefit of glutamine supplementation has been difficult in individual studies. Powell-Tuck et al[70] performed a double blind, randomized, controlled trial of glutamine supplementation in parenteral nutrition with a total of 168 patients who had been referred for parenteral nutrition. However

despite this large study no difference in infective complications or in length of hospital stay could be demonstrated. There was, however, a reduction in length of hospital stay, improved immune status and nitrogen economy shown by Powell-Tuck for the sub-group of patients post-op major abdominal surgery[71].

1.5.2 Glutamine treatment in neonatal catabolic states

1.5.2.1 Neonatal Animal studies

It has previously been shown that mediators of sepsis (e.g. hydrogen peroxide and nitric oxide) inhibit mitochondrial metabolism in neonatal suckling rat hepatocytes[72,73], and that these effects could be reversed by glutamine through its effects on the synthesis of glutathione, an important intracellular antioxidant[74]. This reversal of mitochondrial inhibition was shown to be exclusive to glutamine and did not occur with other amino acids[74]. This shows that the antioxidant effect of glutathione is being boosted by increased production of glutathione from glutamine. The glutathione therefore appears to be providing a protective effect possibly by scavenging free radicals produced in the inflammatory process.

It has also been shown that hepatocytes from endotoxaemic neonatal rat pups also had decreased mitochondrial metabolism and damaged mitochondrial ultrastructure, and that this could similarly be reversed by incubation of hepatocytes *in vitro* with glutamine[75]. Endotoxaemic neonatal rat pup hepatocytes have decreased glutamine levels compared to controls[76] and the recovery seen in hepatocytes incubated with

glutamine may be due to restoration of glutamine levels and increased ATP synthesis by the endotoxic rat hepatocytes[75].

1.5.2.2 Neonatal Infant studies

Studies in preterm infants have recently been reviewed by the Cochrane collaboration[77]. The primary outcomes of death prior to hospital discharge (relative risk 0.98, 95% CI 0.80-1.21) and neurodevelopmental outcome (30 month data still awaited) did not show any difference between the glutamine and no glutamine groups. Secondary outcomes of systemic infection, necrotising enterocolitis, weight gain, days to establishing enteral feeds and days from birth to discharge home from hospital did not show any significant statistical difference on meta-analysis. Sub-group analysis of administration either enterally or parenterally did not show any difference between glutamine and no glutamine groups. Tubman et al therefore concluded that there is no evidence from good quality controlled trials to suggest that the routine use of parenteral or enteral glutamine supplementation alters clinical outcomes in very low birth weight infants[77]. They hypothesized that that any benefits of glutamine similar to that seen in adult studies may be confined to critically ill infants e.g. with sepsis or necrotising enterocolitis. Glutamine supplementation may be beneficial in the recovery phase of these illnesses when infants are severely metabolically compromised and glutamine availability may be the rate-limiting step for tissue repair. There is also an ongoing study of "Glutamine supplementation in parenteral nutrition of surgical infants" (Surgical Infants Glutamine Nutrition (SIGN) Trial. Prof. Agostino Pierro, Institute of Child Health, 30 Guilford St, London WC1N) for which we await results.

2 Aim

The aim of this work was to investigate the effect of glutamine administration in vivo in order to ascertain whether some of the beneficial effects of glutamine, seen when glutamine was administered to endotoxic cells, could be replicated in vivo under more physiological conditions in a neonatal rat model.

Furthermore this work was aimed to assess the clinical effects of *in vivo* glutamine administration to see if any of the *in vitro* effects of glutamine seen in previous work could be replicated. For example, previous work had shown changes in oxygen consumption in endotoxic hepatocytes that could be partially corrected by administration of glutamine *in vitro*. The aim of my work was to assess whether any of these changes could be replicated in an *in vivo* model of endotoxaemia in conjunction with *in vivo* administration of glutamine.

Also assessment of whether these effects seen *in vitro* would have any clinically apparent benefit in a whole animal model of sepsis and glutamine administration. Clinical implications of glutamine administration are important, as the overall aim of the research is to assess whether glutamine administration could be beneficial in a neonatal septic clinical situation as an adjunct to treatment in order to help prevent morbidity and mortality due to organ failure.

3 Endotoxaemia Model

3.1 Introduction

In order to study the effect of glutamine on sepsis in neonatal animals a model of sepsis was used. This involved the induction of endotoxaemia in neonatal rats that had been developed, previously, at Institute for Child Health, London[75]. Glutamine has been suggested to be beneficial in sepsis in *in vitro* studies and therefore administration of glutamine *in vivo* was studied. Glutamine utilization in sepsis increases and therefore any effects of glutamine could either be because of a pharmacological effect of glutamine administration or else it could be that there is a depletion of glutamine due to sepsis or endotoxaemia that is prevented or treated by administration of glutamine. Measurement of the plasma concentrations of glutamine in an endotoxaemia model of neonatal sepsis would therefore help to assess the changes in plasma glutamine concentration that are occurring in endotoxaemia in those administered glutamine compared to those not administered glutamine.

3.2 Hypothesis

Hypothesis that glutamine administration in endotoxic neonatal rat pups would improve their clinical condition. Development of a neonatal animal model of

endotoxaemia with administration of glutamine as a therapeutic measure was needed to assess the hypothesis. Assessment is needed of plasma glutamine levels in neonatal endotoxic rats and with those administered glutamine intraperitoneally. Assessment of the effects of glutamine administration on the clinical condition of endotoxic neonatal rat pups including rectal temperature, power and mobility is needed to test the initial hypothesis.

3.3 Methods

3.3.1 Animals

11-13 day old Wistar rat pups were used. These were obtained from Harlan Sera-Lab, Loughborough, Leicestershire, and kept 10 pups per mother

3.3.2 Home office personal licence

This work was carried out under Personal Licence PIL 70/15656 of the Home Office in accordance with the Animals (Scientific Procedures) Act 1986.

3.3.3 Home office project licence

Home Office Project Licence PPL 70/4590 held under Professor L. Spitz and Professor A. Pierro at the Institute for Child Health, 30 Guilford Street, London WC1N 1EH.

3.3.4 Suckling Rat Endotoxaemia

To model neonatal sepsis, we used a model of endotoxaemia in suckling rats. This model gives a reproducible sepsis-like condition[75]. *E.Coli* endotoxin was injected into suckling rats to induce an inflammatory cascade similar to sepsis. Ten rat pups per litter were split into two groups of five. A single intraperitoneal injection was given to each rat pup of 0.9% Saline with or without lipopolysaccharide (*E.Coli) Sigma* (LPS 05:B55). The concentration of lipopolysaccharide was 12.5μg/ml of injection and the volume of injection was 0.024ml/g of body weight. This gave a dose of LPS of 300μg/Kg bodyweight. The control group (C) had 0.9% saline only. The endotoxaemia group (E) had LPS dissolved in 0.9% saline. There were also further groups where L-glutamine was dissolved in the injection solution at a concentration to 12.175μg/ml before injection. These were the control glutamine group (CG) (0.9% Saline + Glutamine) and the endotoxaemia glutamine group (EG) (0.9% Saline + LPS + Glutamine).

Two further groups received L-leucine dissolved in to the injection solution at an isomolar concentration to glutamine. These two groups were the control leucine group (CL) (0.9% Saline + Leucine) and the endotoxaemia leucine group (EL) (0.9% Saline + LPS + Leucine). Hence all groups received 0.024ml/kg.

3.3.5 Rectal temperature

Rectal temperature was monitored hourly using an aqueous jelly lubricated temperature probe inserted 1-1.5cm in to the rectum. Rectal temperatures less than 32°C were defined as being hypothermic.

3.3.6 Body weight

Body weight was measured hourly to check for signs of dehydration. If body weight dropped by more than 10% then the rat pups were sacrificed to prevent unnecessary suffering.

3.3.7 Endotoxaemia score

The rat pups were then monitored for the following signs of endotoxaemia using a modified[75] score of Morton and Griffiths[78](Figure 5).

Figure 5 Table of score of endotoxaemia

Mobility

0- the rat pups will not allow themselves to be supine

2- the rat pups can be turned supine for a few seconds

4- the rat pups can be turned supine for more than 5 seconds

6- the rat pups will not turn back over when turned supine

Power

0- the rat pups can dangle from a finger for more than 5 seconds

1- the rat pups can dangle from a finger for a few seconds

2- the rat pups cannot dangle from a finger

Shivering

0- no shivering

1- shivering

The score for shivering is additional. If the total score is 8 for mobility and power then shivering is not counted: i.e. max score of 8 (Very ill); minimum score of 0 (no signs of illness).

3.3.8 Sacrifice of animal

At time intervals of either 2, 4 or 6 hours post injection the rat pups were sacrificed by Schedule 1 method of cervical dislocation. Blood was collected by decapitation from each group of rat pups in to a lithium-heparin tube, which was centrifuged and the plasma (supernatant) was removed and stored in a freezer at –18°C.

3.3.9 Glutamine assay

Glutamine was measured spectrophotometrically[79]. This was done in two stages. Firstly, glutamine was de-aminated by glutaminase (*E. Coli*) to produce glutamate. Then the glutamate was de-aminated by glutamate dehydrogenase (GlDH) in a reaction with oxidised nicotinamide adenine dinucleotide (NAD) to produce α-ketoglutarate and NADH.

Chemical Equations

L-Glutamine + H_2O ---glutaminase→ L-glutamate + NH_4^+

L-Glutamate + NAD^+ + H_2O ---GlDH→ α-ketoglutarate + NH_4^+ + NADH

The increase in NADH concentration, measured by the change in absorbance at 340nm is proportional to the amount of glutamate. The samples are tested for concentration of glutamate without enzymatic hydrolysis of glutamine to glutamate and then are tested post enzymatic hydrolysis of glutamine to glutamate. The two concentrations are then subtracted from each other to give the concentration of glutamine in the initial sample.

Optimisation of conditions

The glutaminase reaction proceeds optimally by using acetate buffer (0.5M) to maintain a pH of 5.0. Under normal physiological conditions and concentrations, the glutamate dehydrogenase reaction favours the formation of glutamate. Therefore, to overcome the unfavourable kinetics a number of steps were taken. A high concentration of NAD^+ was used along with a low proton concentration pH 9.0 by using a Tris/hydrazine buffer (Tris 0.1M, EDTA 2mM, hydrazine 0.63M pH 9.0). Furthermore, hydrazine in the buffer acts as a trapping agent for α-ketoglutarate. Ammonium (NH_4^+) free enzyme preparations were used in the glutamate assay. ADP was also included in this assay to maximally activate glutamate dehydrogenase (GlDH) and to decrease its reactivity towards other amino acids.

Deproteinization

An equal volume of plasma sample and perchloric acid solution (10% w/v) were mixed and centrifuged for 3min at 3000g. The supernatant was then neutralised with KOH solution (20% w/v) and then placed in an ice bath for 10min. The precipitated potassium perchlorate ($KClO_4$) is then removed by centrifuging and the supernatant retained.

Enzymatic hydrolysis by glutaminase

For the enzymatic hydrolysis of glutamine to glutamate by glutaminase, 0.1ml of deproteinised plasma sample was mixed with 0.2ml acetate buffer (0.5M), 0.01ml glutaminase solution (10kU/l) and 0.69ml water before incubating for 1 hour at 37°C.

Determination of glutamate

Determination of glutamate was performed twice for each plasma sample, once after deproteinization (glutamate) and once after deproteinization and enzymatic hydrolysis (glutamine + glutamate). In each case 1.0ml Tris/hydrazine buffer (see above) was mixed with 0.1ml NAD solution (β-NAD 30mM), 0.01ml ADP solution (ADP 100mM) and 0.79ml water. To this was added 0.1ml of sample either post deproteinization or post deproteinization and glutaminase enzymatic hydrolysis. After mixing thoroughly the absorbance was measured at 340nm (reading A1) before adding 0.02ml glutamate dehydrogenase (GlDH) and then incubating for 40min. After incubation the absorbance was re-measured until constant (reading A2). Readings A2 – A1 = ΔA.

Calculations

The increased absorbance being measured was that of NADH as it is produced by glutamate dehydrogenase (GlDH).

Change in absorbance ΔA = concentration x extinction coefficient ($6.22 mM^{-1}cm^{-1}$) x path length

From this formula, and with adjustments for the dilution effect of the reactions, the concentration of glutamine and glutamate combined and the concentration of glutamate alone can be calculated for each sample.

Glutamine concentration = (Conc. of glutamine plus glutamate) – (Conc. of glutamate)

Standard solutions of glutamine were used to check this spectrophotometric measurement of glutamine.

3.3.10 Statistical Analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical comparison of multiple groups was performed by One-way ANOVA with Newman-Keuls Multiple Comparison post-test. Prism 3.02 and Instat 3.05 (both GraphPad Software, San Diego California USA) were used for statistical comparisons.

3.4 Results

3.4.1 Clinical Observations

3.4.1.1 Rat Body weight

Rat body weight was measured hourly during all experiments as an assessment of dehydration of the rat pups. A reduction in weight of greater than 10% body weight warranted sacrifice to prevent suffering with dehydration. All measurements showed less than 5% change in weight. No rats were sacrificed due to weight loss.

3.4.1.2 Endotoxaemia score

From our modified endotoxaemia score a range of severity of effect of endotoxaemia could be ascertained from 0 (no symptoms) to a maximum of 8 (very sick). Measurement of endotoxaemia score was made at hourly intervals following the injection of the rat pups. Each day of experiment the endotoxaemia

scores of the 5 rat pups in each group at each time point are averaged. Following intraperitoneal injection of endotoxin (LPS), the endotoxin group (E) and the groups injected with endotoxin plus either glutamine (EG) or leucine (EL) had an increased sepsis score over time (Figure 6A). Rats injected with endotoxin plus glutamine, however, showed a decreased sepsis score throughout the time course; this was significantly different to the endotoxin or endotoxin plus leucine groups at 150 and 210 minutes. The difference seen in the endotoxin plus glutamine group was about 2 points below the endotoxin group and the endotoxin plus leucine group. All the control groups had endotoxaemia scores of less than two throughout the time course post injection. Thus the endotoxin plus glutamine group, though significantly less than the endotoxin and endotoxin plus leucine groups, was still very significantly sick compared to the control groups.

At 150min post injection One-way ANOVA p=0.0003.

Newman-Keuls Multiple Comparison Test	P value
Endotoxin Glutamine *vs.* Endotoxin Leucine	$P < 0.001$
Endotoxin Glutamine *vs.* Endotoxin	$P < 0.01$
Endotoxin *vs.* Endotoxin Leucine	$P > 0.05$

At 210min post injection One-way ANOVA $p<0.0001$

Newman-Keuls Multiple Comparison Test	P value
Endotoxin Glutamine *vs.* Endotoxin	$P < 0.001$
Endotoxin Glutamine *vs.* Endotoxin Leucine	$P < 0.001$
Endotoxin *vs.* Endotoxin Leucine	$P > 0.05$

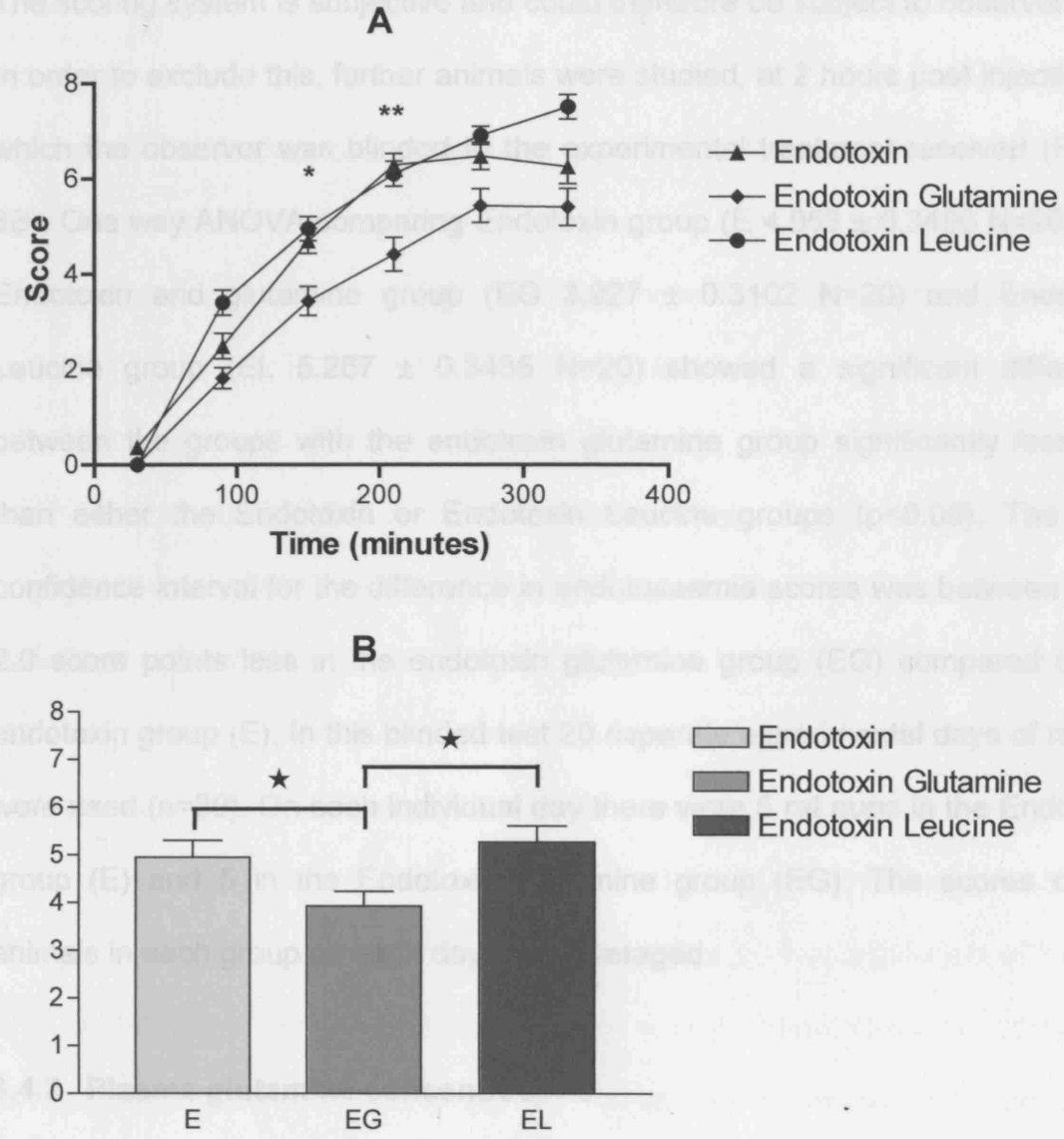


Figure 6 **Effect of glutamine on clinical score in endotoxaemia**

A Average endotoxaemia score *vs.* Time
* EG *vs.* E $p<0.01$; EG *vs.* EL $p<0.001$ N=40
** EG *vs.* E; EG *vs.* EL $p<0.001$ N=40

B Observer blinded endotoxaemia scores at 120 min
One way ANOVA $p<0.02$
E *vs.* EG, EG *vs.* EL *$p<0.05$
E group (Mean ± SEM) 5.0 ± 0.3 N=20
EG group (Mean ± SEM) 3.9 ± 0.3 N=20
EL group (Mean ± SEM) 5.3 ± 0.3 N=20

The scoring system is subjective and could therefore be subject to observer bias. In order to exclude this, further animals were studied, at 2 hours post injection, in which the observer was blinded to the experimental treatment received (Figure 6B). One way ANOVA comparing Endotoxin group (E 4.953 ± 0.3480 N=20) with Endotoxin and glutamine group (EG 3.927 ± 0.3102 N=20) and Endotoxin Leucine group (EL 5.267 ± 0.3435 N=20) showed a significant difference between the groups with the endotoxin glutamine group significantly less sick than either the Endotoxin or Endotoxin Leucine groups ($p<0.05$). The 95% confidence interval for the difference in endotoxaemia scores was between 0.1 – 2.0 score points less in the endotoxin glutamine group (EG) compared to the endotoxin group (E). In this blinded test 20 separate experimental days of results were used (n=20). On each individual day there were 5 rat pups in the Endotoxin group (E) and 5 in the Endotoxin glutamine group (EG). The scores of the animals in each group on each day were averaged.

3.4.2 Plasma glutamine concentrations

2 hours post injection, plasma glutamine concentration was significantly ($p<0.001$) reduced in the endotoxin group (E=0.32 ±0.06mM, n=10) compared to the control group (C=0.73±0.06mM). The control glutamine group (CG=0.82±0.07mM) was significantly higher ($p<0.05$) than the endotoxin glutamine group (EG=0.59±0.04mM), however neither CG nor EG groups were significantly different from the control group (C *vs.* CG or EG $p>0.05$). EG had a significant increase in plasma glutamine concentration compared to E group

($p<0.01$). (Figure 7A) (One-way ANOVA with Newman-Keuls Multiple Comparison Test)

4 hours post injection, plasma glutamine concentration in the endotoxin group (E=0.63±0.10mmol/l) had recovered compared to 2 hours post injection. There was no significant difference between the groups at 4 hours (One-way ANOVA $p=0.07$, C=0.74±0.06, E=0.63±0.10, CG=0.85±0.03, EG=0.86±0.06mmol/l)(Figure 7B). Comparing the plasma glutamine concentrations at 2hrs and at 4hrs, there was no significant difference between control (C) groups (C 2hr=0.73±0.06, C4hr=0.74±0.06, $p>0.05$) nor between the control glutamine (CG) groups (CG 2hr=0.82±0.07, CG 4hr=0.85±0.03, $p>0.05$) (One-way ANOVA with Newman-Keuls Multiple Comparison Test). Between the endotoxin (E) groups at 2hrs and 4hrs there was a significant increase in plasma glutamine levels (E 2hr=0.32±0.06, E 4hr=0.63±0.10, $p<0.01$). In the endotoxin plus glutamine (EG) group at 2hrs and at 4hrs there was no significant difference from control (C) (C *vs.* EG $p>0.05$ at 2hours or at 4 hours). However, compared to the control glutamine (CG) there was a decrease in plasma glutamine in the endotoxin glutamine (EG) group at 2hrs ($p<0.05$), which by 4hrs post injection had recovered (CG 4hr *vs.* EG 4hr $p>0.05$). This increase in endotoxin glutamine (EG) plasma glutamine levels at 4 hours compared to 2 hours (EG 2hr=0.59±0.04, EG 4hr=0.86±0.06, $p<0.05$) was statistically significant.

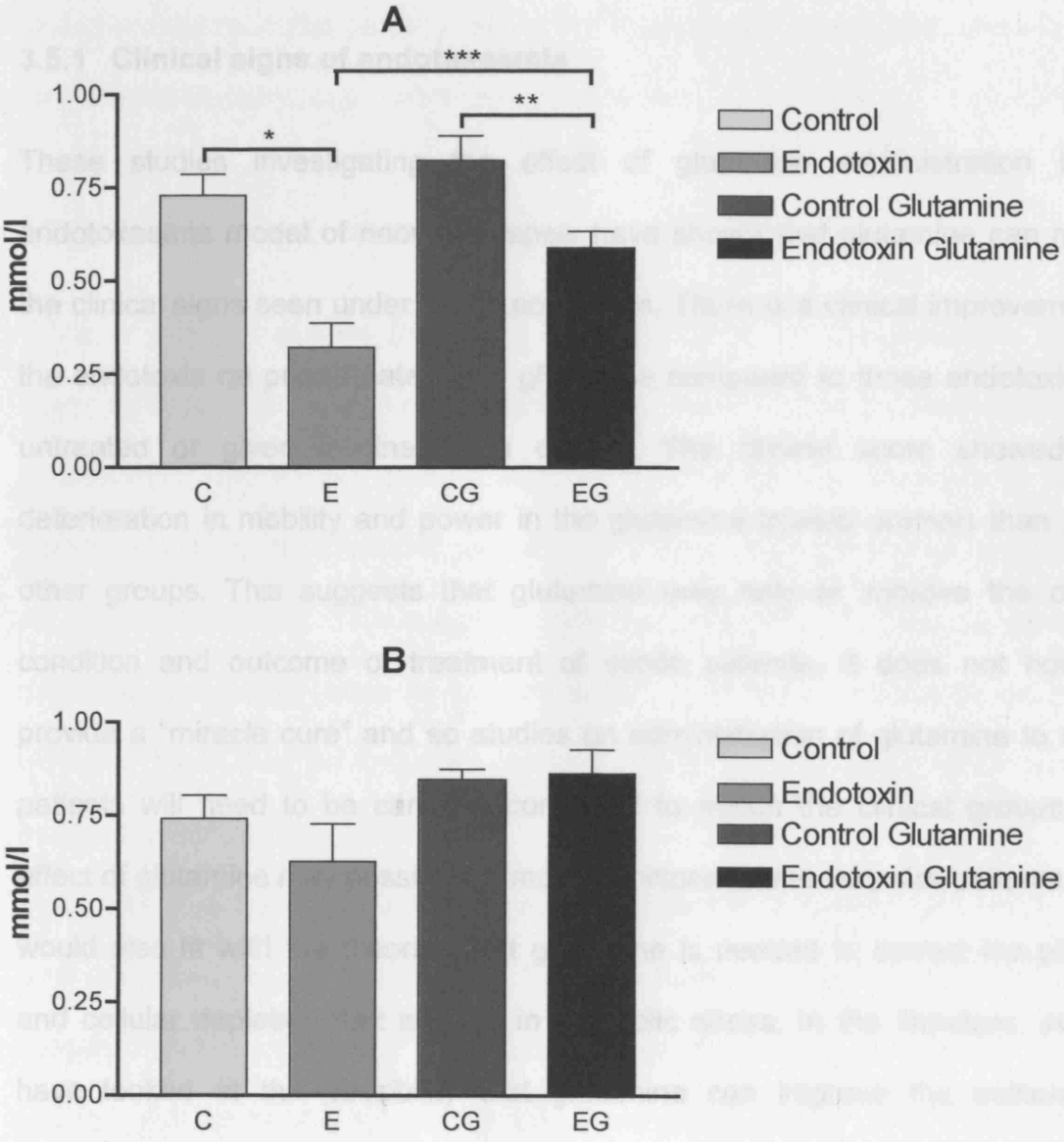


Figure 7 Plasma glutamine levels

A Plasma glutamine levels at 2 hours post injection (n=5)
* $p<0.001$ Control *vs.* Endotoxin
** $p<0.05$ Control Glutamine *vs.* Endotoxin Glutamine
*** $p<0.01$ Endotoxin *vs.* Endotoxin Glutamine

B Plasma glutamine levels at 4 hours post injection (n=5)

3.5 Discussion

3.5.1 Clinical signs of endotoxaemia

These studies investigating the effect of glutamine administration in an endotoxaemia model of neonatal sepsis have shown that glutamine can modify the clinical signs seen under septic conditions. There is a clinical improvement in the endotoxic rat pups treated with glutamine compared to those endotoxic rats untreated or given leucine as a control. The clinical score showed less deterioration in mobility and power in the glutamine treated animals than in the other groups. This suggests that glutamine may help to improve the clinical condition and outcome of treatment of septic patients. It does not however provide a "miracle cure" and so studies on administration of glutamine to septic patients will need to be carefully controlled to match the clinical groups. The effect of glutamine may possibly be more significant in the very sick patients. This would also fit with the theories that glutamine is needed to correct the plasma and cellular depletion that is seen in catabolic stress. In the literature, studies have looked at the possibility that glutamine can improve the outcome of intensive care patients by theoretically reducing the incidence of sepsis and the severity of infection[80,81,82]. A recent meta-analysis has confirmed that glutamine does reduce infection rates and length of hospital stay in adults. However, a Cochrane Review of glutamine in very low birth weight premature neonates has not shown any benefit of glutamine administration either by enteral or parenteral nutrition[77] as a method for prevention of infection. While my experiments suggest

that glutamine may be beneficial in sepsis, it is not clear how glutamine is causing this benefit, whether this is related to decreased bacterial translocation and improved mucosal barrier function, or to improved metabolism and an effect on the inflammatory response in endotoxaemia.

3.5.2 Plasma glutamine levels

While in previous studies there has been a drop in plasma glutamine seen in septic patients[83] there has not been shown to be a definite glutamine deficiency in humans[57]. My results show that there is a temporary decrease in plasma glutamine initially, however this recovers by 4 hours post injection in this model of acute endotoxaemia. This suggests that replacement of total body glutamine depletion is not the cause of the effects seen on administration of glutamine. However there may be localised glutamine depletion that could be boosted by early administration of glutamine.

Furukawa et al have shown that there is a decrease in plasma glutamine levels post-operatively compared to controls[44]. Neutrophils isolated from patients after surgery[84], burns or trauma[85] had been shown to have decreased bactericidal activity. Furukawa et al showed that the bactericidal activity could be restored *in vitro* if the neutrophils were incubated in a glutamine supplemented solution. The most significant increase in bactericidal activity back towards control neutrophil activity levels was shown to occur with an increase in glutamine concentration from 0.5mmol/l to 1mmol/l[44]. In plasma glutamine levels that I measured the endotoxaemic group had plasma glutamine levels of 0.32mmol/l at 2 hours post

injection. This is a level consistent with impaired neutrophil bactericidal activity in the endotoxin group and recovery of at least some of that activity in the endotoxin glutamine group (EG). By four hours post injection the endotoxaemic group had mobilised glutamine synthesis and/or release to increase levels back to 0.63mmol/l. The glutamine supplemented endotoxaemic group (EG) showed a far smaller fall in plasma glutamine levels (0.59mmol/l at 2 hours compared with control 0.73mmol/l). By 4 hours post injection the EG group had increased the plasma glutamine levels to 0.86mmol/l. Though this was higher than control plasma glutamine levels there was not a statistical difference between the control group and the EG group.

These results suggest that the initial decrease in plasma glutamine levels is caused by an increased utilisation of glutamine by some cells in the body that then stimulates the animal to mobilise glutamine reserves either by breakdown of muscle protein or by synthesis of glutamine. Muscle glutamine reserves are very large, and presumably it would be necessary to prolong the experiments much further before muscle glutamine depletion is observed. If this is the case then the local plasma glutamine levels in the vicinity of cells with increased utilisation of glutamine might well be even lower than the measured plasma levels. For example, in septic rats enterocytes have been shown to have increased glutamine consumption[86]. The intestines, because of their high glutamine requirement in sepsis, are particularly prone to the effects of reduced plasma glutamine. Other cells that have a high glutamine requirement include the immune system. In the glutamine treated group there is a markedly reduced

decrease in plasma glutamine compared with that seen in those endotoxic rat pups not treated with glutamine.

In summary, short-term plasma glutamine depletion is seen that can be at least partially restored by administration of glutamine. The glutamine levels measured in the endotoxin group (E) are also of the similar levels to those shown previously to have a deleterious effect on neutrophil activity. While the glutamine concentration in the glutamine supplemented groups are higher and are consistent with recovery of neutrophil bactericidal activity. The effects of glutamine on the inflammatory and immune system were further examined in Chapter 8.

4 Effects on isolated hepatocytes

4.1 Introduction

The liver is the first organ to be affected by sepsis from the intestinal tract via the portal circulation. Previous work at the Institute of Child Health showed that there was a decrease in isolated hepatocyte oxygen consumption when incubated with hydrogen peroxide[72] and nitric oxide[73]. Hydrogen peroxide and nitric oxide are possible mediators of septic damage and was used therefore as a model for sepsis. Using the model of sepsis described in Chapter 3.3, with endotoxin administration, a reduction in mitochondrial oxygen consumption of isolated hepatocytes was less marked in those incubated with glutamine compared to without[75]. Assessment of *in vivo* glutamine administration in endotoxaemia on hepatocyte metabolism and function is needed to see if the effects in vitro can be reproduced in an *in vivo* setting.

4.2 Hypothesis

The hypothesis was that *in vivo* glutamine administration in neonatal endotoxaemia would improve the oxygen metabolism of hepatocytes that is reduced in endotoxaemia.

4.3 Methods

4.3.1 Isolation of hepatocytes

Rat pups were treated using the model of endotoxaemia, described in Chapter 3, with or without administration of glutamine. The hepatocytes were then isolated by the following method[73]. At 2 hours post intra-peritoneal injection the rat pups were sacrificed by a schedule 1 method and blood was taken by decapitation. The livers were then harvested into Krebs Ringer Phosphate solution (see section 4.3.1.1). The livers were washed in KRP solution and finely chopped into approximately $2mm^3$ pieces before being re-washed with KRP to remove any blood. The liver pieces were re-suspended in 20ml Krebs Ringer Bicarbonate solution (KRB (see section 4.3.1.1)) + 1% (weight/volume) Bovine Serum Albumin (BSA) in a 100ml conical flask. 0.5mM EGTA (200µl of 40mM) was added and the suspension was gassed with a 95% O_2, 5% CO_2, before being sealed with a subaseal cap.

The liver was then incubated at 37°C in a water bath, while being agitated at approximately 150 shakes per minute for 30 minutes. Removal of Calcium by EGTA helps to disrupt cellular tight junctions. The liver/hepatocyte suspension was re-gassed every 15 minutes throughout the rest of the isolation. After 30 minutes 2mM calcium chloride ($CaCl_2$) (0.5 ml of 80mM), 0.05% of collagenase (0.01g) and 0.0025% DNAse (50 µL of 1% w/v solution) were added and the

suspension was re-gassed and resealed and then incubated for a further 30 min in the water bath. Ca^{2+} ions are necessary for the action of collagenase.

At the end of the incubation the supernatant was poured off and centrifuged at 400rpm for 2 minutes. The pellets from the centrifuge tubes and the liver remaining from the flasks were then re-suspended in 20ml KRB +1% BSA. 2mM Calcium Chloride ($CaCl_2$) (0.5 ml (500μL) of 80mM), 0.05% of collagenase (0.01g) and 0.0025% DNAse (50 μL of 1% w/v solution) were added and the suspension was re-gassed and resealed. The suspension was then incubated for a further 40min at 37°C in a shaking water bath. The suspension was re-gassed at 10 minute intervals.

At the end of the incubation the contents of the flasks were poured onto a nylon 100micron mesh and gently massaged through the mesh, breaking up the remaining liver in the process. The cells were then washed through the mesh with the Washing solution (see section 4.3.1.1). The suspension produced was poured in to two 50ml centrifuge tubes and topped up with washing solution. The suspension was then centrifuged at 400 rpm for 2 minutes and the supernatant was then discarded. The pellets were then re-suspended in washing solution and re-centrifuged at 400rpm for 2 minutes. The pellet was re-suspended in Medium solution (see section 4.3.1.1) in a 100ml conical flask and stored in the shaking water bath at 37°C. This suspension of isolated hepatocytes was then gassed every 15 minutes.

4.3.1.1 Solutions

Krebs Ringer Phosphate solution (KRP)

8g NaCl, 0.4g KCl, 0.2g $MgSO_4.7H_2O$, 0.12g Na_2HPO_4, 0.02g KH_2PO_4, made up to 1000ml with distilled water.

Krebs Ringer Buffered solution (KRB) pH 7.3-7.4.

8g NaCl, 0.4g KCl, 0.2g $MgSO_4.7H_2O$, 0.12g Na_2HPO_4, 0.02g KH_2PO_4, 0.025mol $NaHCO_3$ made up to 1000ml with distilled water.

Hepatocyte Medium Solution

150ml KRB, 3g BSA, 0.044g $CaCl_2$, 0.024g L-Carnitine

Washing Solution

500ml KRB, 0.145g $CaCl_2$,

4.3.2 Viability of hepatocytes

The viability of the hepatocytes was assessed using a haemocytometer and trypan blue staining. 100μl of cell solution was mixed with an equal volume of trypan blue solution (0.15% w/v in saline). With trypan blue, dead or damaged cells take up the stain and are coloured blue, while live healthy cells do not take up the trypan blue and so remain clear. The number of viable cells was calculated as a percentage of the total number of cells (viable and non-viable). Only isolated hepatocyte suspensions with a viability of greater than 85% were used.

4.3.3 Dry weight of isolated hepatocytes

1ml of isolated hepatocyte suspension was put into two previously weighed micro-centrifuge tubes and then dried in a 60°C oven for 2 hours. Each tube was

then re-weighed and the dry weight of cells per ml of solution was calculated from the average weight of cells in the two tubes. This dry weight was then used to standardise the results of experiments from isolated hepatocytes.

4.3.4 Hepatocyte oxygen consumption measurement with Clark type electrode

O_2 consumption of isolated hepatocytes and hepatocyte mitochondria were measured using a Clark type electrode[72]. This consists of a platinum electrode and a reference electrode of silver immersed in a potassium chloride (KCl) solution. When a voltage of -0.6V is connected across the two electrodes immersed in an oxygen containing solution, with the platinum electrode negative relative to the reference electrode, oxygen undergoes an electrolytic reduction. Oxygen from the medium diffuses across the membrane and is reduced at the electrode to hydroxide ions (OH^-). The hydroxide ions can then diffuse through the potassium chloride solution to the silver electrode. The size of the current passing to the silver electrode is therefore proportional to the amount of oxygen reduced per unit time and this depends on the rate of diffusion of oxygen across the membrane. For thin membranes and low levels of dissolved oxygen, the rate of diffusion is directly proportional to the activity of oxygen in the solution surrounding the membrane. The relationship between the current and the oxygen concentration is directly proportional at a polarisation voltage of –0.6 volts.

Reference oxygen concentration levels are obtained at 0% oxygen by saturating with sodium dithionite ($Na_2S_2O_4$). Air saturated concentration of oxygen in

solution at 37°C and 1 atmosphere pressure is measured by fully saturating KRB solution with air. Air saturated water solutions at 37°C and 1 atmosphere pressure have 0.215 micromoles of oxygen per millilitre.[87]

4.3.5 Oxygen consumption in isolated hepatocytes

Isolated hepatocytes were incubated for 15min prior to measurement in a mixture made up of 1ml of cell suspension, 2ml of medium (see section 4.3.1.1) and 1ml of palmitate solution (KRB + 2% BSA (w/v) + 2mM palmitate. Palmitate was dissolved into solution using a ultrasonic bath.

This was then added to the electrode chamber and the oxygen concentration was measured against time to give a rate of consumption of oxygen by the cells at 37^0C. This was related to the dry weight of the same volume of cell suspension to give a result of oxygen consumption per gram of dry weight of hepatocytes. Calibration of the electrode was performed daily using sodium dithionite ($Na_2S_2O_4$) to give zero oxygen and air saturated KRB solution (0.215micromoles O_2/millilitre). Oxygen consumption was then calculated assuming a linear correlation between oxygen consumption and electrode voltage under these conditions.

Once consistent oxygen consumption was attained, a saturating concentration (10ng/ml) of oligomycin, an inhibitor of the phosphorylation pathway, was added and the oxygen consumption in the presence of oligomycin was measured. Oligomycin blocks the intramitochondrial phosphorylation by complex V (ATP-synthetase) of ADP to form ATP that is then used as the energy source for many

cellular reactions. Oligomycin inhibits the proton channel part of complex V. This therefore blocks the use of complexes I, III and IV of the respiratory chain by preventing use of the proton gradient across the inner mitochondrial membrane (Figure 8). Intramitochondrial oxygen consumption used for phosphorylation of ADP to ATP can therefore be calculated by subtracting the oxygen consumption in the presence of oligomycin from the oxygen consumption in the absence of oligomycin (total cellular oxygen consumption).

Results are quoted in nanomoles of oxygen consumption per minute per mg dry weight of cells (nmol/min/mg dry weight).

4.3.6 Statistical Analysis

Results are expressed as mean ± standard error of the mean (SEM). Unpaired Student t-test was used for comparison of the Endotoxin minus Control group and Endotoxin Glutamine minus Control Glutamine group. Statistical comparison of multiple groups was performed by One-way ANOVA with Newman-Keuls Multiple Comparison post-test. Prism 3.02 and Instat 3.05 (both GraphPad Software, San Diego California USA) were used for statistical comparisons.

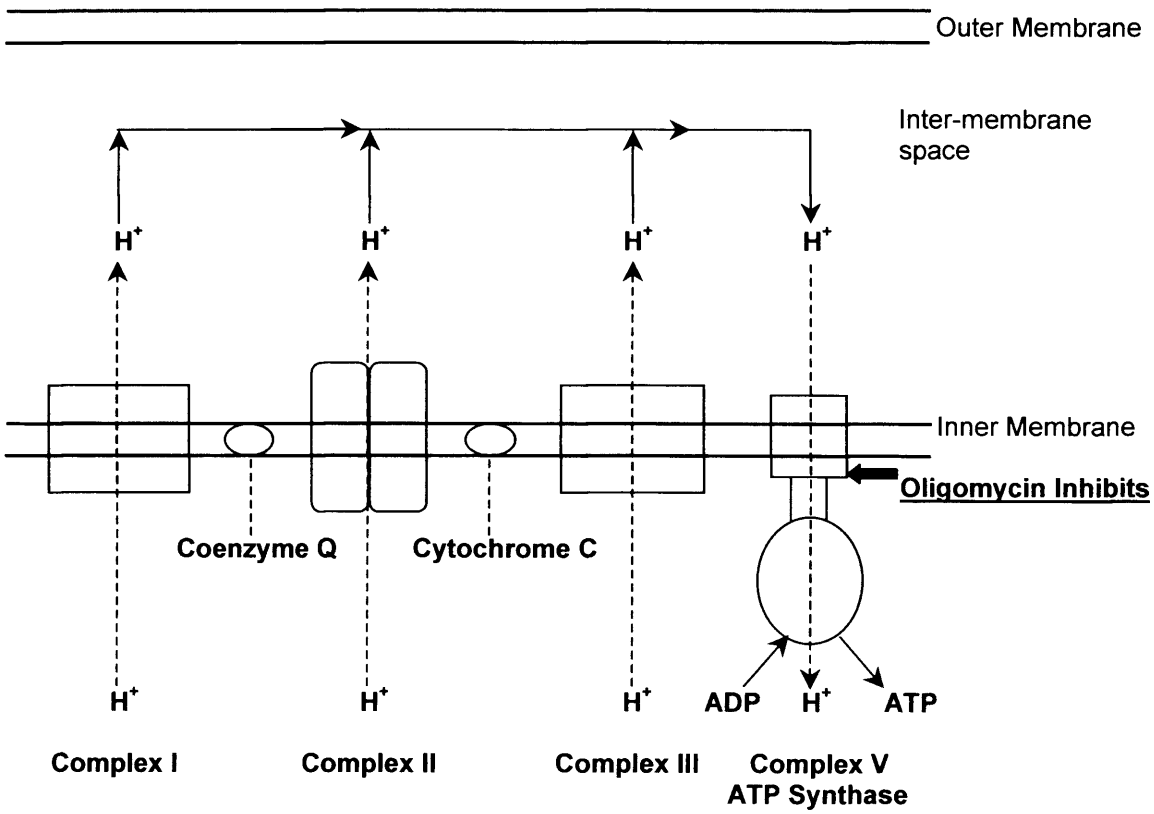


Figure 8 Respiratory chain proton gradients

4.4 Results

4.4.1 Isolated hepatocyte oxygen consumption

Isolated neonatal rat hepatocytes from each of the four groups (Control, Endotoxin, Control Glutamine, and Endotoxin Glutamine) were incubated with palmitate solution and oxygen consumption measured with a Clark type electrode.

The control (C) and endotoxin (E) groups were both taken from one litter of rats on each day of experiments. The control glutamine (CG) and endotoxin glutamine (EG) groups were similarly both from one litter of rats on each day of experiments. There was a slight decrease in oxygen consumption between control (C) and endotoxin (E) groups (C=2.06±0.14, E=1.82±0.11nmol O_2/min/mg dry weight). However, between control glutamine and endotoxin glutamine groups there was a slight increase in oxygen consumption (CG=1.81±0.12, EG=1.94±0.14nmol O_2/min/mg dry weight). These were not however statistically significant on testing by One-way ANOVA (p=0.45)(Figure 9A).

If the results from each litter of rats were taken together, as the difference between endotoxin (E) and control (C) groups or the difference between endotoxin glutamine (EG) and control glutamine (CG) groups (Figure 9B). Then there is a significant difference between those treated with glutamine compared to those not given glutamine (p=0.04 E-C=-0.2458 ± 0.1153 N=16, EG-

CG=0.1343 ± 0.1349 N=16). This shows a possible increase in oxygen consumption with glutamine administration in endotoxaemia. This assumes that part of the day to day variability in results is due to differences in timing etc in the lengthy process of isolation of hepatocytes. As each day involved only two groups, due to the practical difficulties in isolation of the hepatocytes, comparing the difference between the two groups on each day could therefore factor out differences due to the variation in each day's isolation procedure. Either Control and Endotoxin groups or Control Glutamine and Endotoxin Glutamine groups are therefore paired. The difference between the paired groups for each day is used for analysis. These results show that there is an increase in oxygen consumption of isolated hepatocytes in the endotoxin Glutamine group (EG) compared to the Control Glutamine group (CG) while there was a decrease in the Endotoxin group's (E) oxygen consumption compared to the Control group (C). (Figure 9B)

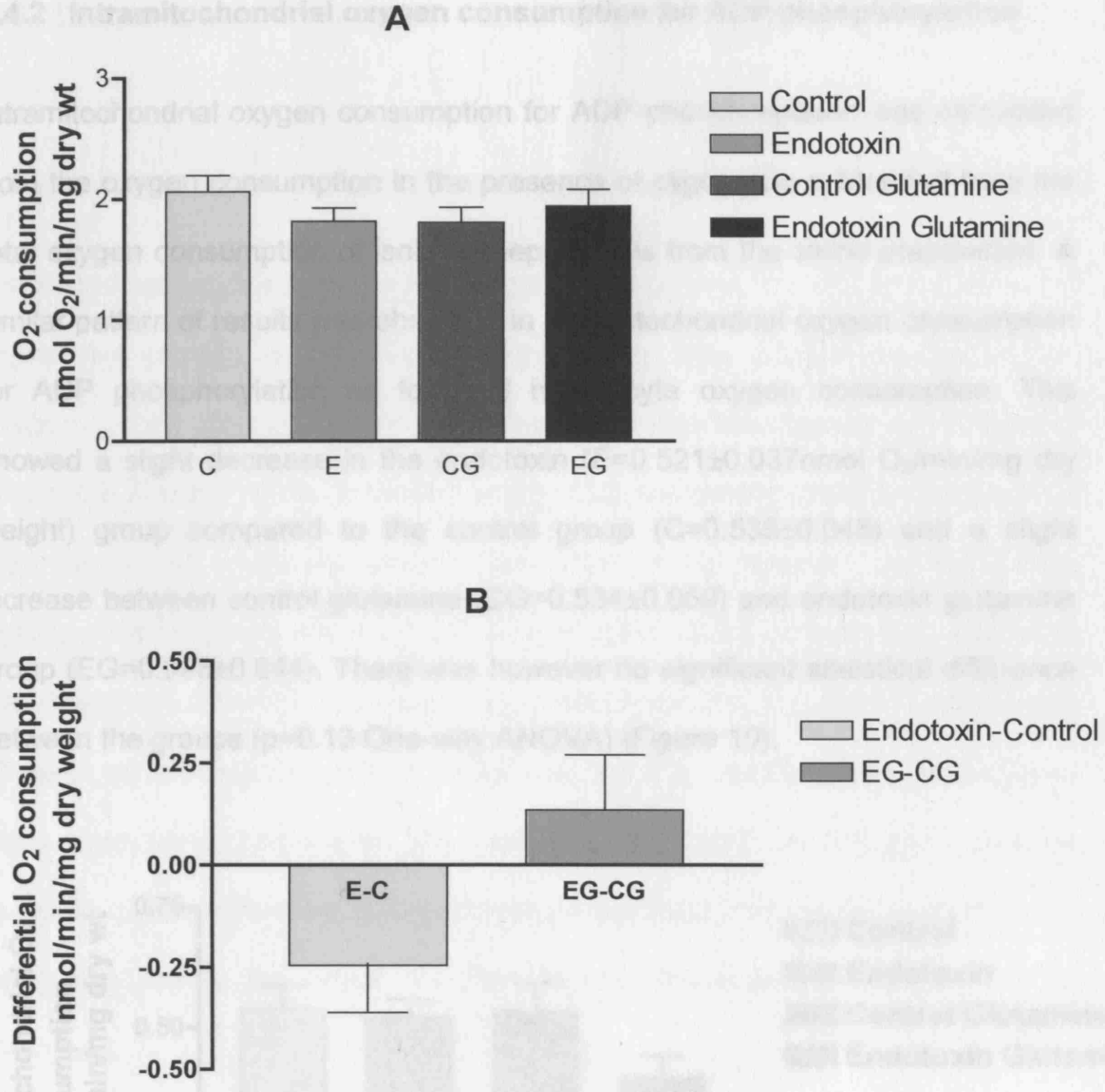


Figure 9 **Oxygen consumption of isolated hepatocytes**

A Control 2.06±0.14 (n=16) nmol/min/mg dry weight
Endotoxin 1.82±0.11 (n=16)
Control Glutamine 1.81±0.12 (n=16)
Endotoxin Glutamine 1.94±0.14 (n=16)

B Differential Oxygen consumption (n=16)
Endotoxin minus Control -0.25±0.12
Endotoxin Glutamine - Control Glutamine 0.13±0.15
p=0.04 unpaired t test

4.4.2 Intramitochondrial oxygen consumption for ADP phosphorylation

Intramitochondrial oxygen consumption for ADP phosphorylation was calculated from the oxygen consumption in the presence of oligomycin subtracted from the total oxygen consumption of isolated hepatocytes from the same preparation. A similar pattern of results was observed in intramitochondrial oxygen consumption for ADP phosphorylation as for total hepatocyte oxygen consumption. This showed a slight decrease in the endotoxin (E=0.521±0.037nmol O_2/min/mg dry weight) group compared to the control group (C=0.535±0.048) and a slight increase between control glutamine (CG=0.534±0.059) and endotoxin glutamine group (EG=0.398±0.044). There was however no significant statistical difference between the groups (p=0.13 One-way ANOVA) (Figure 10).

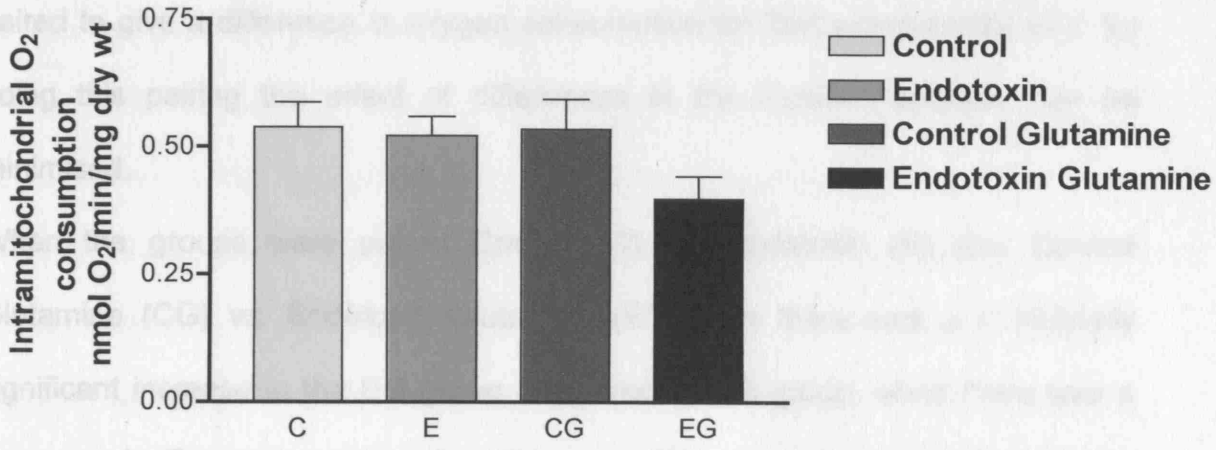


Figure 10 Mitochondrial oxygen consumption isolated hepatocytes

Control	0.54±0.05 nmol O2/min/mg dry wt (n=16)
Endotoxin	0.52±0.04 nmol O2/min/mg dry wt (n=16)
Control Glutamine	0.53±0.06 nmol O2/min/mg dry wt (n=16)
Endotoxin Glutamine	0.40±0.04 nmol O2/min/mg dry wt (n=16)

p=0.13 One-way ANOVA

4.5 Discussion

4.5.1 Isolated neonatal hepatocyte oxygen consumption

I isolated hepatocytes from the four experimental groups in order to measure the hepatocyte oxygen consumption using a Clark type oxygen electrode. The oxygen consumption of the endotoxin group (E) was less than its paired control group (C). The endotoxin glutamine group (EG) had greater oxygen consumption than the control glutamine group (CG). There were, however, no statistically significant differences shown if all groups were taken in isolation. Technical difficulties in maintaining consistent percentage viability of isolated hepatocytes between experimental days has meant that the only statistically significant data came when each experimental day's two groups (C and E or CG and EG) were paired to give a difference in oxygen consumption for that experimental day. By doing this pairing the effect of differences in the isolation process can be minimised.

When the groups were paired Control (C) *vs.* Endotoxin (E) and Control Glutamine (CG) *vs.* Endotoxin Glutamine (EG) then there was a statistically significant increase in the EG group compared to CG group, while there was a decrease in E group compared to C group. This suggests that the endotoxin group have a reduced oxygen consumption of isolated hepatocytes compared to controls while the Endotoxin glutamine group has increased oxygen consumption. This does however rely on the assumption that that the two control groups will have similar oxygen consumption or that the control glutamine (CG)

group has increased oxygen consumption compared to the control group (C). Though this would seem reasonable, as glutamine is an energy source for the citric acid cycle, it is in no way certain that glutamine, with its many metabolic pathways, may not have the entirely opposite effect of decreasing the oxygen consumption of the CG group compared to C group.

The changes measured of oxygen consumption in isolated hepatocytes were however smaller in magnitude than those seen in *in vitro* experiments[75]. This is to be expected as in *in vitro* administration of glutamine the control group receives no glutamine supplementation at the equivalent time. While in this *in vivo* administration of glutamine at the time of intraperitoneal injection of endotoxin the control group will have a normal plasma glutamine level and not a zero plasma glutamine concentration.

The isolation process is however a long process and during this time the cells of all groups have no glutamine supplementation in the bathing solution. This means that the isolation process itself might affect the viability and oxygen consumption at the time of harvest of the liver. However it could also be that there is only a minimal change in the hepatocyte oxygen metabolism. Markley et al had previously shown that there was no significant difference in hepatocyte oxygen consumption in isolated endotoxaemic hepatocytes treated with or without glutamine *in vitro*. However, Markley et al did show that in endotoxaemia there was a decrease in hepatocyte intramitochondrial oxygen consumption at the same time as an increase in the extramitochondrial hepatocyte oxygen consumption[75]. The decrease in intramitochondrial oxygen consumption was

restored by the administration of glutamine *in vitro*. In order to avoid changes occurring due to the process of isolation of hepatocytes other methods of assessing intramitochondrial hepatocyte function are needed.

Glutamine may become conditionally essential under septic or endotoxic conditions. Glutamine deficiency could lead to changes in metabolism. Previous work by Babu et al[74] showed that, *in vitro*, glutamine administration could counteract a decrease in hepatocyte oxygen metabolism. He also showed that this effect was controlled by glutathione production from glutamine. It is speculated that glutathione is acting as an antioxidant and this prevents damage by oxygen free radicals on the hepatocyte. However, in these *in vivo* experiments it has been difficult to replicate these effects that were seen *in vitro*.

5 Effects on isolated liver mitochondria

5.1 Introduction

Indirect measurement of intramitochondrial liver oxygen consumption by subtracting the oxygen consumption of cells incubated with oligomycin from the oxygen consumption without oligomycin is likely to introduce variability into the results due to the method of measurement. Direct measurement of isolated liver mitochondria may give improved consistency in results. The isolation process of liver mitochondria is also a much shorter process than the 6 hour hepatocyte isolation procedure. During this isolation time the cell metabolism and glutamine levels may be changing. In the shorter liver mitochondrial isolation process there is less possibility for changes in mitochondrial metabolism and mitochondrial glutamine concentrations.

5.2 Hypothesis

Hypothesis that direct measurement of oxygen consumption in isolated hepatocyte mitochondria will give more consistent results than measurement of hepatocyte oxygen consumption and then calculating intramitochondrial from this and oxygen consumption after administration of oligomycin.

5.3 Methods

5.3.1 Liver Mitochondrial isolation

Liver was obtained from schedule 1 sacrificed rat pups. This was put into 4°C Medium B (250mM sucrose, 2mM Hepes, 0.1mM EGTA pH 7.4 @25°C) and homogenised in a glass hand homogeniser. The homogenised suspension was then centrifuged at 3000rpm for 10min at 0-5°C. The supernatant was placed in a fresh tube while the pellet was re-suspended in Medium B and re-centrifuged at 3000rpm for 10min at 0-5°C. The supernatant from this second centrifugation was placed in a fresh tube and the pellet was discarded.

The supernatant samples were then centrifuged at 13500rpm for 10min. The pellet from this was reserved and the supernatant was discarded. The pellets were re-suspended in micro-centrifuge tubes and re-spun once more at 13500rpm.

The pellet from this was then visually checked for erythrocytes and further centrifuged as necessary to remove erythrocytes. The pellet was then re-suspended in a small volume of medium B to make a mitochondrial suspension of approximately 80-100mg protein/ml[88].

5.3.2 Citrate Synthase Assay

Citrate synthase activity was measured as an indication of the intactness of isolated mitochondria as citrate synthase is found in the mitochondrial matrix. It

was also used to standardise the amount of mitochondria in each sample being tested. A spectrophotometer was set to 412nm and zeroed against a reagent blank at 25^0C. To a 2ml cuvette was added:

965µl 100mM Tris-HCl pH8.0

10µl Acetyl CoA 5mM

10µl 5,5-dithiobis-2-nitrobenzoic acid (DTNB) 10mM

10µl 1:100 mitochondrial solution diluted in medium B

A baseline reading was then obtained, then 5µl of 50mM oxaloacetic acid (OAA) solution was added. (33mg OAA + 250 µl 2M $NaHCO_3$ then 50 µl of this solution was diluted in 950µl Tris HCl). A linear rate was obtained for approximately 3 minutes before 10µl 10%(v/v) Triton-X 100 was added and then run for a few further minutes[89]. The rate after OAA but before Triton X-100 represents disrupted mitochondria, while the rate after Triton X-100 represents total mitochondria.

5.3.3 Calculation of citrate synthase activity

Rate of Absorb/min (after Triton-X100) – rate of absorb/min (after OAA) = rate of citrate synthase activity of intact mitochondria.

Beer-Lambert Law and Calculations:

Absorbance = Extinction Coefficient x Concentration x path length (10mm)

= $mol^{-1}cm^{-1}$ x M x 1cm

Extinction Coefficient for DTNB to DTB at 412nm E= 14600 $mol^{-1}cm^{-1}$

From the Beer-Lambert Law the international units (U) of citrate synthase activity can be calculated where:

1U = 1 μmol product /min

5.3.4 Calculation of mitochondrial intactness (%)

Citrate synthase is found exclusively in the mitochondrial matrix. Citrate synthase activity can therefore be used to calculate the mitochondrial intactness of a sample of isolated mitochondria. Initial measurement of the citrate synthase activity will only measure the activity of mitochondria that are disrupted. This can when compared to the activity when all the sample mitochondria are disrupted (after Triton-X100 administration) can be used to calculate the percent intact initially.

$$\text{Intactness (\%)} = \frac{\text{Absorb/min (after Triton-X100)} - \text{absorb/min (after OAA)}}{\text{Absorb/min (after Triton-X100)}} \times 100$$

The calculated intactness was greater than 99% in all samples measured.

5.3.5 Oxygen consumption in isolated liver mitochondria

Oxygen consumption was also measured in isolated liver mitochondria. A buffered solution of 110mM potassium chloride (KCl), 10mM HEPES, 2.5mM phosphate, 1mM EDTA, 5mM $MgCl_2$, at pH 7.2 was defrosted. *Cytochrome c* 0.2mg/ml was added and warmed in a water bath to 30°C. Having measured citrate synthase activity in the isolated mitochondria, 10μl of mitochondrial suspension was added to 750μl of buffer. Oxygen consumption was measured

using a Clark Oxygen Electrode kept at 30°C with a water jacket. This gave State I mitochondrial oxygen consumption.

7.5μl of 1M glutamate plus 100mM malate was then added to give State II oxygen consumption. Once a steady reading had been obtained 133nmol ADP was added to give State III oxygen consumption in the presence of abundant ADP (Figure 11). Abundant ADP is necessary to prevent the build up of hydrogen ions (H^+) in the intermembrane space that would therefore reduce the rate of oxygen consumption by Complex IV of the respiratory chain. The reaction of ADP and phosphate by ATP synthase uses the hydrogen ion gradient across the inner mitochondrial membrane thus reducing the concentration gradient of H^+ ions against which the reduction of oxygen by Complex IV is working. State III is therefore in the presence of abundant substrate and of abundant ADP. The rate-limiting step is thus due to the maximum capacity of the respiratory chain.

When there is no ADP left and the oxygen consumption as a result tails off then this is State IV oxygen consumption in low or absence of ADP (Figure 11). In this situation the thermogenic proton leak, or any ATP that is being hydrolysed to ADP will be the only factors dissipating the H+ gradient.

	State I	State II	State III	State IV
Characteristics	Aerobic	Aerobic	Aerobic	Aerobic
ADP level	Low	High	High	Low
Substrate level	Low (endogenous)	Approaching zero	High	High
Respiration rate	Slow	Slow	Slow	Slow
Rate limiting component	Phosphate acceptor (ADP)	Substrate	Respiratory Chain	Phosphate acceptor (ADP)

Figure 11 Mitochondrial energy substrates
Table of conditions for mitochondrial energy states.[90]

5.3.6 Complex I Assay

Complex I was assayed as the rotenone-sensitive oxidation of NADH by ubiquinone-1. As complex I is blocked by rotenone, the oxidation of NADH to NAD^+ in the inner mitochondrial membrane can be measured before and after addition of rotenone to give the total NADH rate of reduction and the rate of reduction of NADH while complex I is blocked. Complex I activity can therefore be calculated from the difference between these two measurements[91]. Citrate synthase activity is relatively constant in mitochondria. It is therefore a good measure of the relative concentrations of intact mitochondria in a preparation of isolated mitochondria.

Isolated mitochondria, having measured citrate synthase activity, were diluted in 25mM potassium dihydrogen orthophosphate (KH_2PO_4), 5mM magnesium chloride ($MgCl_2$) pH 7.2 (hypotonic medium) and freeze-thawed three times in liquid nitrogen to break the mitochondrial membranes so that the inner

mitochondrial membrane and therefore the respiratory chain are accessible for reactions.

Reaction buffer is made up of 25mM potassium dihydrogen orthophosphate (KH_2PO_4), 5mM magnesium chloride ($MgCl_2$) pH 7.2 with 2.5mg/ml bovine serum albumin and 3μl per ml 1M potassium cyanide (KCN). The potassium cyanide inhibits Complex IV.

To 975μl of buffer were added 1μl 50mM ubiquinone-1, 1μl 1mg/ml antimycin-A, to inhibit complex III, and 10μl 12.5mM NADH. This is allowed to equilibrate and a baseline absorbance was measured by spectrophotometry at 340nm. 10μl of diluted and freeze-thawed mitochondrial sample solution was then added and the decrease in absorbance at 340nm was measured for 3-4 min to get a linear, measurable rate of the oxidation of NADH. Then, 3μl of 1mg/ml rotenone was added and the rate of oxidation of NADH is measured in the presence of rotenone (Figure 12).

Antimycin-A is an inhibitor of Complex III and KCN is an inhibitor of Complex IV so they are added to reduce any effect due to these complexes. The major non-Complex I activity is NADH b_5 reductase of the mitochondrial outer membrane.

5.3.7 Complex I activity Calculation

Complex I activity is attributed to the rate which is sensitive to rotenone inhibition. This can therefore be calculated by taking the total rate of oxidation of NADH and subtracting the rate of oxidation of NADH insensitive to rotenone (i.e. not inhibited by rotenone). (Figure 12)

Change in Absorbance per minute = concentration of NADH oxidised per minute ($mM.min^{-1}$) x Extinction coefficient ($U.mM^{-1}cm^{-1}$) x path length (cm)

The extinction coefficient for the NADH at 340nm wavelength and 30°C is 6.22 $U.mM^{-1}cm^{-1}$.

Complex I activity (U) is defined as activity to convert 1 μmol/min of NADH.

This in our experiment is divided by the Citrate Synthase (CS) activity (U) of the isolated mitochondria to give a proportional rate of Complex I activity per Citrate synthase activity unit.

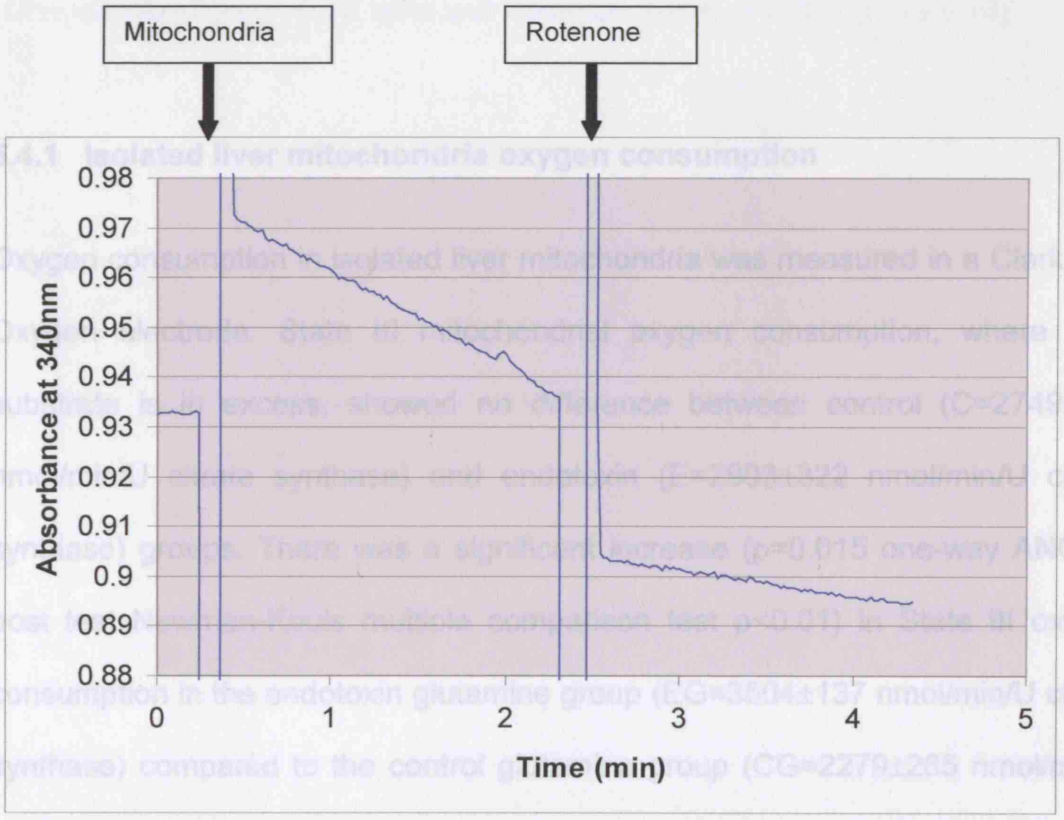


Figure 12 Example graph of NADH oxidation used to calculate Complex I

5.3.8 Statistical Analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical comparison of two groups was performed with the Student t-test. Statistical comparison of multiple groups was performed by One-way ANOVA with Newman-Keuls Multiple Comparison post-test. Prism 3.02 and Instat 3.05 (both GraphPad Software, San Diego California USA) were used for statistical comparisons.

5.4 Results

5.4.1 Isolated liver mitochondria oxygen consumption

Oxygen consumption in isolated liver mitochondria was measured in a Clark type Oxygen electrode. State III mitochondrial oxygen consumption, where ADP substrate is in excess, showed no difference between control (C=2749±193 nmol/min/U citrate synthase) and endotoxin (E=2903±322 nmol/min/U citrate synthase) groups. There was a significant increase ($p=0.015$ one-way ANOVA, post test Newman-Keuls multiple comparison test $p<0.01$) in State III oxygen consumption in the endotoxin glutamine group (EG=3504±137 nmol/min/U citrate synthase) compared to the control glutamine group (CG=2279±265 nmol/min/U citrate synthase). (Figure 13A)

State IV oxygen consumption in the absence of ADP showed a similar pattern of results to State III. However statistically there was no significant difference

between any of the groups (C=574±46, E=655±80, CG=428±46, EG=631±99 nmol/min/U citrate synthase (n=6), p=0.14). (Figure 13B)

5.4.2 Isolated liver mitochondrial Complex I

Complex I activity in isolated mitochondria showed an increase in complex I activity in endotoxin (E=36.0±5.6 mU/U CS, n=8) and endotoxin glutamine groups (EG=39.4±5.8 mU/U CS), relative to citrate synthase activity, compared to their respective controls (C=27.3±5.7 mU/U CS, CG=33.6±5.4 mU/U CS). However this was not significantly different except between EG and C groups (One way ANOVA p=0.04, post test Newman-Keuls $p<0.05$). (Figure 14)

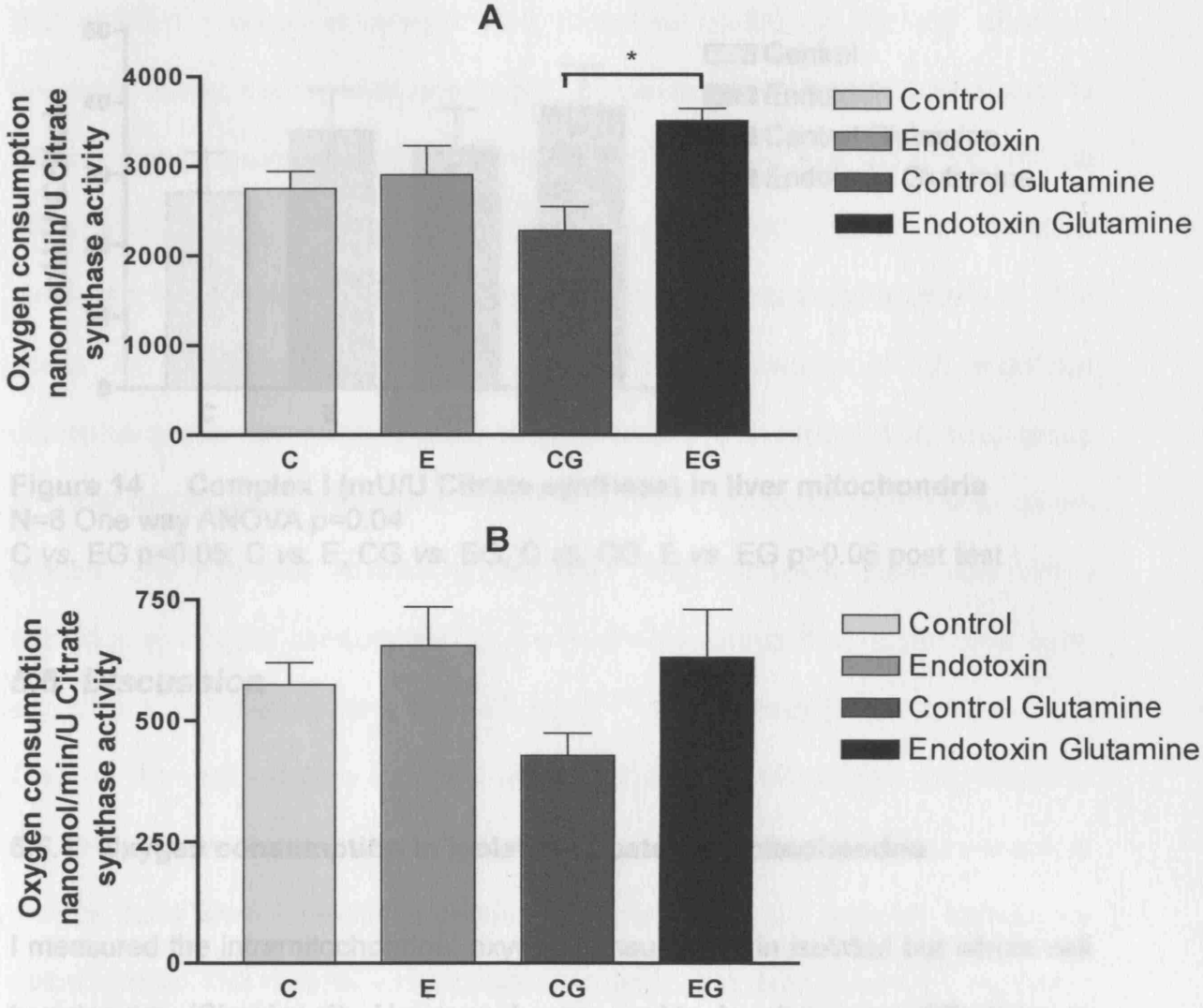


Figure 13 Isolated hepatocyte mitochondria oxygen consumption
A State III isolated hepatocyte mitochondria oxygen consumption
N=6 One-way ANOVA p=0.015 * p<0.01 CG *vs.* EG post-test
B State IV isolated hepatocyte mitochondria oxygen consumption
N=6 One-way ANOVA p=0.14

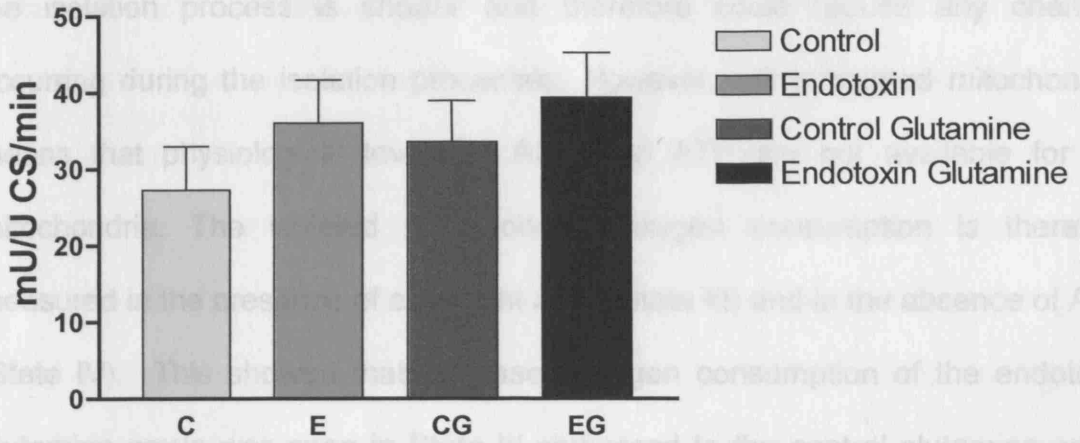


Figure 14 Complex I (mU/U Citrate synthase) in liver mitochondria
N=8 One way ANOVA p=0.04
C *vs.* EG $p<0.05$; C *vs.* E, CG *vs.* EG, C *vs.* CG, E *vs.* EG $p>0.05$ post test

5.5 Discussion

5.5.1 Oxygen consumption in isolated hepatocyte mitochondria

I measured the intramitochondrial oxygen consumption in isolated but whole cell hepatocytes (Chapter 4). However I was unable to show any difference in intramitochondrial oxygen consumption, which may have been because of variability due to the lengthy hepatocyte isolation process. Previous work had shown a decrease in intramitochondrial oxygen consumption in isolated endotoxic hepatocytes[75] that was restored by the *in vitro* administration of glutamine.

By using isolated mitochondria, I attempted to elucidate further the differences in hepatocyte mitochondria oxygen consumption between the groups. One of the

benefits of using isolated mitochondria compared to isolated hepatocytes is that the isolation process is shorter and therefore could reduce any changes occurring during the isolation processes. However, using isolated mitochondria means that physiological levels of ADP and ATP are not available for the mitochondria. The isolated mitochondrial oxygen consumption is therefore measured in the presence of abundant ADP (State III) and in the absence of ADP (State IV). This showed that increased oxygen consumption of the endotoxin glutamine group was seen in State III compared to the control glutamine group and that there was no difference in state IV oxygen consumption between all the groups. The numbers in each group were small, however there was not a decrease in oxygen consumption in the endotoxin group that might have been expected from the previous work by Romeo[72,73] and Markley[75].

One of the main factors involved in changes in mitochondrial respiration is Complex I in the respiratory chain. Complex I can change in activity as a result of various conditions. I therefore measured complex I in the isolated hepatocyte mitochondria. The results, while suggesting an increase in complex I activity in both the endotoxin (E) and endotoxin glutamine (EG) groups did not show any statistical significance compared to the control groups. Induction of a change in complex I activity does not appear to be the method of change in oxygen consumption, though it could still be a partial factor in the changes in oxygen consumption seen.

In all these experiments on hepatocytes and their mitochondria with *in vivo* glutamine administration, I was unable to replicate the statistical differences in

intramitochondrial oxygen consumption seen with the *in vitro* administration of glutamine shown by Markley et al[75]. There is however a fundamental difference between the *in vitro* experiments and the *in vivo* experiments. This difference is that at the time of administration of glutamine to the cells *in vitro* the control group will have zero glutamine compared to the administrated glutamine concentration in the experimental group. However in my *in vivo* experiments the control group has a normal plasma concentration of glutamine, while in the endotoxin group, though it may have a reduced plasma glutamine concentration, its concentration does not fall to zero. Therefore differences due to deficiency in glutamine would only be seen when the glutamine level drops to where glutamine is effectively a "rate limiting step".

6 Glutathione levels in liver and liver mitochondria

6.1 Introduction

Babu et al showed that glutamine prevented a drop in oxygen consumption in isolated hepatocytes incubated in hydrogen peroxide or nitric oxide and that this was mediated through glutathione production from glutamine[74]. Glutamine is needed for the production of glutamate, which is then combined with cysteine and glycine to produce glutathione. Glutathione is an antioxidant that can therefore react with oxygen free radical species that are produced in sepsis. If oxygen free radical species are dissipated by reaction with glutathione this can prevent them from causing further cellular damage and therefore reduce the effects of sepsis or endotoxaemia. Measurements of glutathione concentration in liver and liver mitochondria were made.

6.2 Hypothesis

Hypothesis that glutamine administration in endotoxaemia increases glutathione concentration by acting as a substrate for glutathione synthesis. Increased glutathione concentrations may be beneficial due to its antioxidant effect.

6.3 Methods

6.3.1 Preparation of samples for High Performance Liquid Chromatography

0.9 ml of 10% v/v perchloric acid and 20mM 1,10-phenathroline was mixed with 90μl of either 10mM penicillamine or 10mM gamma glutamyl-glutamate (GGG). Penicillamine and GGG are used as internal standards. Either 100μl of liver mitochondrial solution or approximately 0.1 grams of liver was added to a weighed tube of the above solution and the tube was re-weighed so that the wet weight of liver could be calculated. The liver was homogenised using a glass hand homogeniser and stored on ice. The suspension was then centrifuged at 3000rpm for 10 minutes at 4°C. 250μl of supernatant was removed to a 1.5ml microcentrifuge tube. To this was added 25μl of 20mg/ml iodoacetic acid (freshly prepared) and 125μl of 2M potassium hydroxide (KOH) – 2.4M potassium hydrogen carbonate ($KHCO_3$). The pH was checked to be between pH 8-9. The solution was then vortexed vigorously and incubated for 15-30 minutes in the

dark before adding 500μl of 1% (v/v) flurodinitrobenzene in ethanol. The solution was re-vortexed and allowed to derivatize overnight in the dark.

6.3.2 Glutathione measurement with High Performance Liquid Chromatography

A Jasco-Borwin High Performance Liquid Chromatograph was used to quantify glutathione levels in the liver homogenate and liver mitochondria[92].

A Hypersil APS-2 250mm x 4.6mm column was used with variable solution made up of water (solution A), methanol (solution B), and 0.72M pH4.6 sodium acetate in methanol (solution C). Each sample was run through the column for 50 minutes, followed by a period of washing of the column, before injection of the next sample.

Initial conditions were 19%A, 76%B, 5%C. Following injection of the sample (200μl) at time 0 minutes the conditions were gradually changed through the course of the run to be 13.6%A, 55%B, 31.4%C at time 33 minutes.

Time	A%	B%	C%
0min	19%	76%	5%
33min	13.6%	55%	31.4%
35min	11%	45%	44%
50min	0%	0%	100%
55min	0%	0%	100%
56min	19%	76%	5%

Gamma glutamyl-glutamate (GGG) had a retention time of approx. 38-39min, reduced glutathione (GSH) was 41-42min and oxidised glutathione (GSSG) was 45-46min. Detection of derivatives was at 466nm. Iodoacetic acid blocks free thiol groups and FDNB reacts with amine groups to yield derivatives that absorb at 466nm. Peaks were measured using Borwin Chromatography Software and calculations were made using standards of GSH and GSSG (Sigma, Poole, Dorset, and U.K.). The amounts of GSH and GSSG were calculated using GGG as an internal standard. Total glutathione was then calculated using the formula:

Total Glutathione = GSH + 2 x GSSG

6.3.3 Statistical Analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical comparison of multiple groups was performed by One-way ANOVA with Newman-Keuls Multiple Comparison post-test. Prism 3.02 and Instat 3.05 (both GraphPad Software, San Diego California USA) were used for statistical comparisons.

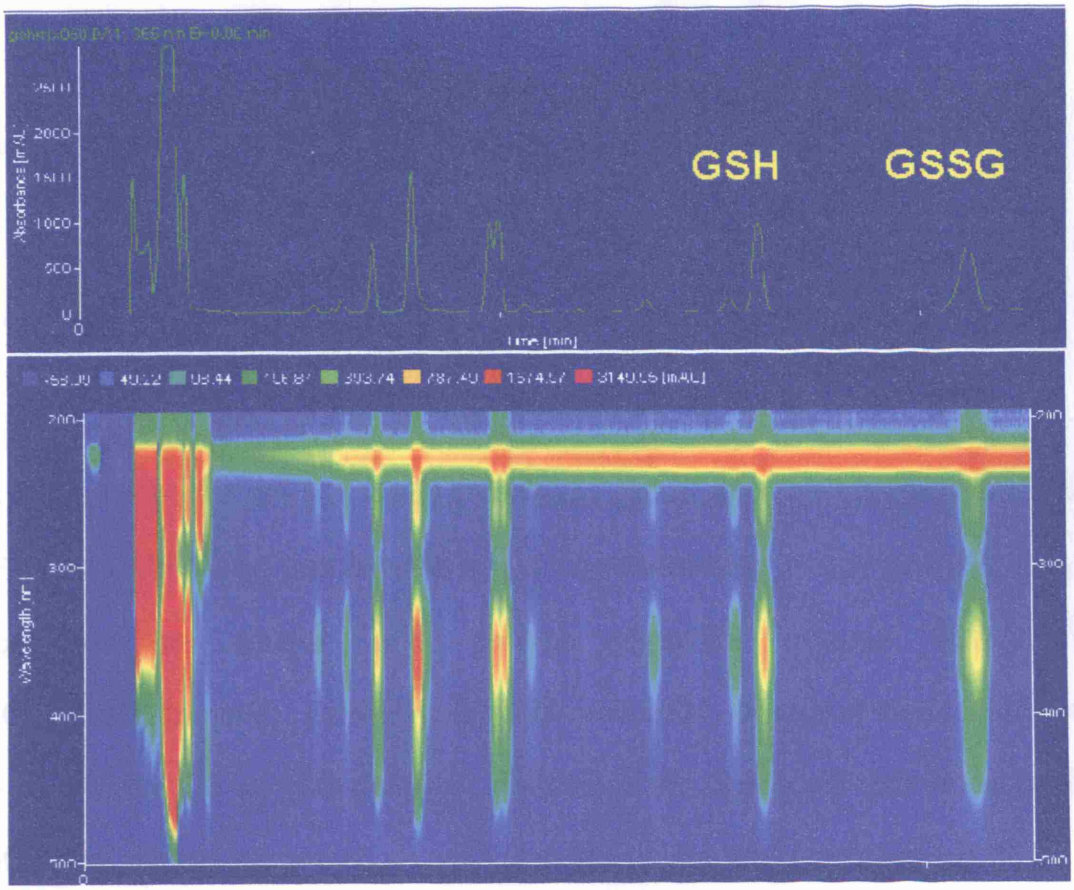


Figure 15 Example HPLC measurement of glutathione

6.4 *Results*

6.4.1 Homogenised liver glutathione concentrations at 2 hours post injection

In both the endotoxin (E=178±11.7nmol/g) group and the endotoxin glutamine (EG=165±8.9nmol/g) group there was a significant drop in liver glutathione levels compared to control and control glutamine groups (C=233±16.0, CG=256±20.1nmol/g). One-way ANOVA $p<0.0001$, post test Newman-Keuls C *vs.* E $p<0.05$, CG *vs.* EG $p<0.001$. There was no significant difference between either the control groups (C *vs.* CG $p>0.05$) or the endotoxin groups (E *vs.* EG $p>0.05$). (Figure 16)

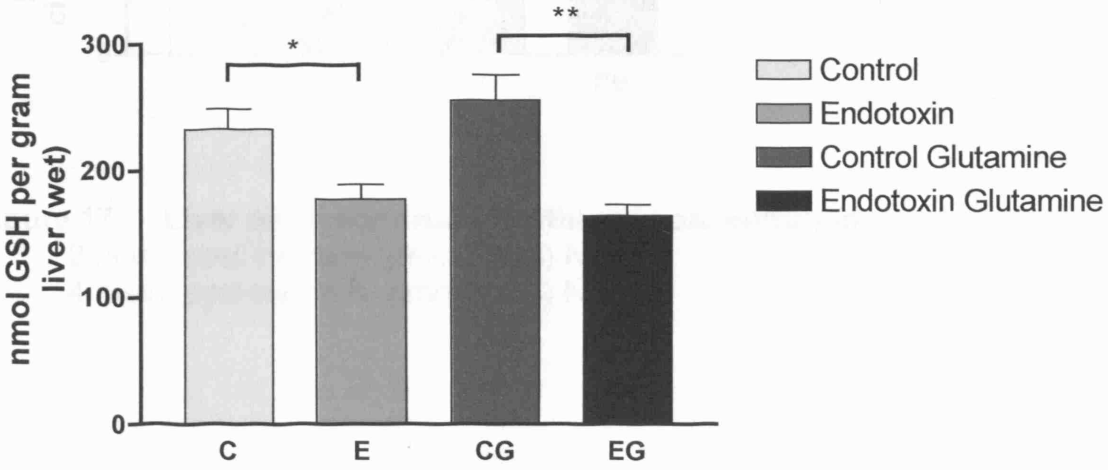


Figure 16 **Homogenised liver glutathione concentration** (nmol GSH/ g liver) N=35
*p<0.05 C *vs.* E, **p<0.001 CG *vs.* EG

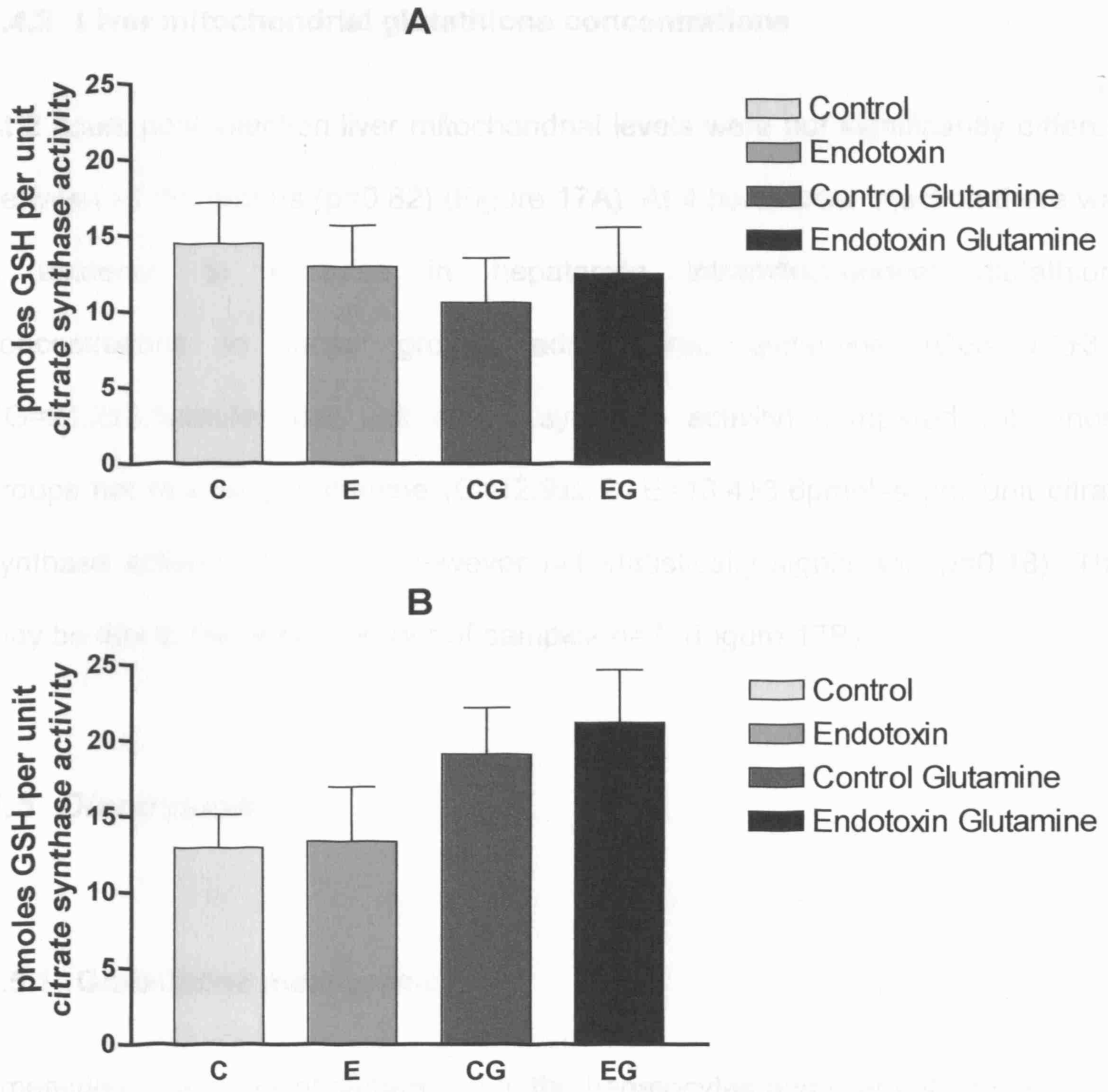


Figure 17 Liver mitochondrial glutathione concentration
A 2 hours post injection (pmol/U CS) N=7
B 4 hours post injection (pmol/U CS) N=7

6.4.2 Liver mitochondrial glutathione concentrations

At 2 hours post injection liver mitochondrial levels were not significantly different between all the groups (p=0.82) (Figure 17A). At 4 hours post injection there was a tendency to increase in hepatocyte intramitochondrial glutathione concentrations in those groups administered glutamine (CG=19.1±3.1, EG=21.2±3.5pmoles per unit citrate synthase activity) compared with those groups not receiving glutamine (C=12.9±2.2, E=13.4±3.6pmoles per unit citrate synthase activity). This was however not statistically significant (p=0.18). This may be due to the small number of samples n=7. (Figure 17B)

6.5 Discussion

6.5.1 Glutathione measurements

I measured the levels of glutathione in the hepatocytes and liver mitochondria, as the liver is a major metaboliser of glutamine. Previous studies have also shown that the inhibitory effects of mediators of sepsis (Nitric Oxide and Hydrogen peroxide) can be reversed by the administration of glutamine in isolated neonatal hepatocytes[72]. It was also shown that this effect was exclusive to glutamine, of all the amino acids[74]. Babu et al also showed that in their isolated rat pup hepatocyte model that the beneficial effect of glutamine was eliminated in the presence of a glutathione synthase inhibitor. This shows that the mechanism of action of glutamine was via glutathione production[74].

In both the endotoxin and endotoxin glutamine groups there was a decrease in glutathione levels in the liver compared to controls ($p<0.0001$) at 2 hours post injection. There was however no difference between the endotoxin (E) and endotoxin glutamine groups (EG). We were not able to measure the flux of glutamine in this neonatal model and therefore the administration of glutamine could be still be acting via glutathione production with an increase production and metabolism of glutathione occurring while the concentrations of glutathione intracellularly remain relatively constant.

Intramitochondrial levels of glutathione were however raised in both the control glutamine (CG) and the endotoxin glutamine groups (EG) compared with the groups that were not administered glutamine. This was not statistically significant though the sample size (n=7) was small. Larger experimental groups may give a statistically significant increase. This could be due to an increased synthesis of glutathione. An increase in intramitochondrial glutathione may be protective to the mitochondria against oxidative stress damage from oxygen free radical species.

Glutathione levels measured were combined reduced glutathione (GSH) and oxidised glutathione (GSSG). Therefore changes in the relative amounts of reduced and oxidised glutathione were not measured. Glutathione has been shown to be an important factor in antioxidant defense which may occur by networking with other antioxidants e.g. vitamin E and vitamin C. Glutathione plays a critical role in regenerating vitamin C and E from their oxidised byproducts[93]. Glutathione levels in other tissues may be boosted by glutamine administration. Glutathione levels have been shown to be important in AIDS therapy as patients with HIV infection have

decreased glutathione in their plasma and leucocytes. This decrease in glutathione was mainly confined within T cells and restoration of the thiol status and glutathione in these cells markedly improved patient survival. I was unable to measure the T cell glutathione levels in neonatal rats[93].

Glutathione levels in animal or human cells may be involved in the mechanism of action of the beneficial effects of glutamine administration in neonatal rat endotoxaemia though I was unable to show a statistical significant change in glutathione levels.

7 Indirect Calorimetry

7.1 Introduction

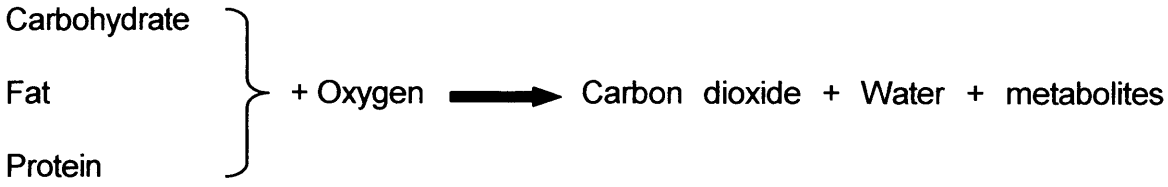
In view of the improvement in clinical observations of the endotoxin glutamine group compared to the endotoxin group, a measurement of the whole animal metabolism and oxygen consumption was made. This was done to determine the overall effect of endotoxaemia on neonatal rat pups as well as the effect of glutamine in endotoxaemia, while avoiding the difficulties in processing and isolating individual tissue samples. Glutamine was hypothesized to be affecting the metabolism of many different tissues in the animal.

7.2 Hypothesis

Hypothesis that the metabolism of endotoxic rat pups is altered compared to controls and that glutamine administration in endotoxaemia may restore the rate of metabolism.

7.3 Methods

Energy for metabolism in humans and animals is obtained from the food that is eaten. Food is made up of carbohydrates, fats and proteins, which are oxidised with oxygen, obtained by the lungs, in cells to produce energy and carbon dioxide. The carbon dioxide is then subsequently excreted via the lungs.



Indirect calorimetry is based on the premise that gas volumes and concentrations exchanged at the mouth reflect cellular metabolic activity. Substrate is oxidised to produce energy and carbon dioxide using oxygen. Complete oxidation of carbohydrate produces 1 mole of carbon dioxide for every one mole of oxygen used. This gives a respiratory quotient (RQ) of 1. Therefore if the amount of oxygen extracted by the lungs is the same as the amount of carbon dioxide produced by the lungs, then it is reasonable to assume that the animal is metabolizing almost exclusively carbohydrate. Fat and protein have respiratory quotients (ratio of CO_2/O_2) of approximately 0.7 and 0.8 respectively. The proportion of each substrate being metabolized can therefore be estimated from the respiratory quotient. Using estimated calorific values for carbohydrate, fat and protein an estimate for the total heat production of the animal(s) can therefore be calculated.

However for indirect calorimetry to give an accurate reflection of whole body metabolism there must be complete oxidation of substrate. Therefore indirect

calorimetry is able to give an accurate measurement of resting energy expenditure. However any situation with incomplete oxidation of substrate is not suitable for measurement of metabolism e.g. during anaerobic respiration in exercise.

By measuring the difference between inspired and expired levels of oxygen and carbon dioxide, determinants of VO_2 and VCO_2 can be obtained. These values are then converted to resting energy expenditure (REE) via a metabolic computer.

An indirect calorimeter was used consisting of a perspex sealed chamber (Columbus Instruments small animal Calorimeter chamber) into which 5 rat pups (Figure 18) at a time were placed for a period of 30 minutes at a time before being returned to the mother for 30 minutes. The chamber had an inflow connection, a sampling connection and an outflow connection. Measurement of the inflow air rate was made as well as the oxygen and carbon dioxide percentage of the inflow air. From the sample port a measurement of the oxygen and carbon dioxide percentage was also made. From these measurements the amount of oxygen consumed and the amount of carbon dioxide produced per kilogram body weight of rat pup per minute was calculated. This gave VO_2 and VCO_2 in millilitres per kilogram per minute.

7.3.1 Indirect Calorimeter

A Columbus Instruments Indirect Calorimeter (Figure 18) was used and was calibrated using Nitrogen gas (N_2) (oxygen and carbon dioxide free) and a mixture of Oxygen, Nitrogen and Carbon dioxide gases at concentrations of 20.5% O_2, 0.5% CO_2 and the remainder N_2 (79.0%). A flow rate of 0.4 l/min was

used through the calorimeter chamber. Measurements were made every minute and then averaged over the 30min that the rat pups were in the indirect calorimeter.

7.3.2 Oxygen sensor

Oxygen sensor was of the limited diffusion, metal air battery type. This contained an anode, electrolyte, and an air cathode to which the diffusion of oxygen is restricted by a diffusion barrier. At the air cathode oxygen is reduced to hydroxyl ions which oxidise the metal anode. The current is proportional to the rate of oxygen consumption. The output leads of the cell are connected across a resistor whose value is chosen so as to operate the cell in the limiting current region i.e. oxygen is being consumed as fast as it reaches the electrode. In this way all oxygen contacting the cathode is consumed and thus the output current is proportional to the oxygen concentration. Knowledge of the resistor's value along with the fact that the cell operates in the limiting current region provides a direct link between oxygen concentration and an observed voltage.

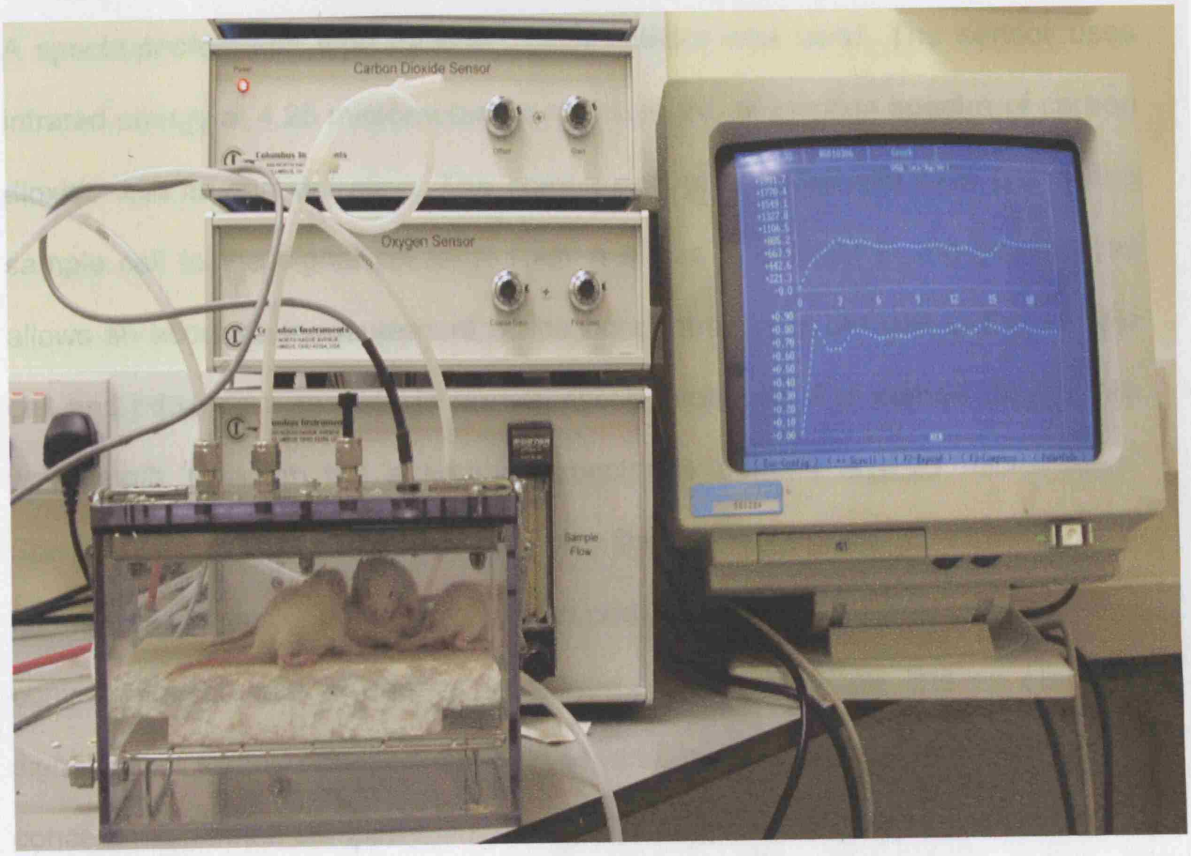


Figure 18 Columbus Instruments Indirect Calorimeter

7.3.3 Carbon dioxide sensor

A spectrophotometric type carbon dioxide sensor was used. The sensor uses infrared energy at 4.25 micrometers to measure the absorption spectra of carbon dioxide with its concentration. The comparison of a signal with zero gas in the sample cell to the signal presented when a gas is present in the sample cell allows an accurate measurement of the concentration of the gas in the sample cell and provides a reading in percentage by volume. For carbon dioxide the wavelength at which the absorption spectra is centred is 4.25 micrometers. Special filtering in the sensor allows only the energy contained within +/- 0.02 micrometers of this centre wavelength to pass to an infrared sensor which in turn converts the amount of energy not absorbed by the sample into an electrical signal. The signal provided by the sample is inversely proportional to the concentration in the sample.

7.3.4 Heat lamp

A 60-watt heat lamp set between 30-50cm away from the indirect calorimeter chamber was used to heat the chamber. The distance of the lamp from the calorimeter was adjusted according to the temperature reading in the calorimeter chamber in order to maintain a constant temperature.

7.3.5 Columbus Instruments' Oxymax system

This software calculated the oxygen consumption and carbon dioxide production in the indirect calorimeter. This was calculated from the flow rate and oxygen or carbon dioxide fraction entering and leaving the chamber. The respiratory quotient is also calculated from this data, which can give an indication of the substrates being used for metabolism. Respiratory quotient is the ratio of oxygen used to carbon dioxide produced. The respiratory quotient therefore varies according to the substrate being metabolised. One mole of Carbohydrate when metabolised fully with one mole of oxygen will produce one mole of carbon dioxide. Metabolism of carbohydrate alone therefore gives a respiratory quotient of one.

Net Carbohydrate metabolism gives an RQ = 1. Net Fat metabolism RQ = 0.71

The Oxymax software can then calculate the energy expenditure using the tabulated calorific values that are tied to respiratory quotient in a manner tabulated by Lusk (1928). For the range of RQ (0.71 to 1.0) the heat available is 4.69 to 5.05 kcal/litre O_2. Energy expenditure can then be calculated using these values and the oxygen consumption (l/min).

7.3.6 Statistical Analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical comparison of two groups was performed with the Student t-test. Statistical comparison of multiple groups was performed by One-way ANOVA with Newman-Keuls Multiple Comparison post-test. Fishers' exact test was used to compare the

proportions of animals hypothermic between the groups. Linear regression analysis was performed to compare temperature with oxygen consumption. Prism 3.02 and Instat 3.05 (both GraphPad Software, San Diego California USA) were used for statistical comparisons.

7.4 Results

7.4.1 Rectal Temperature

Rectal temperature was measured at hourly intervals in all experiments. This corresponded to the end of the 30min of measuring of metabolism by the indirect calorimeter. The normal rectal temperature in suckling rats of this age is 33-35°C[94].

7.4.1.1 Effects of endotoxaemia on rectal temperatures at room temperature

The cage temperature was similar (23.9±0.1°C) in all groups and did not change significantly during the course of the experiments. Rectal temperature dropped in all three groups given intraperitoneal endotoxin (E, EG, EL) 90min post-injection and remained lower than the control groups (C, CG, CL) throughout the experiment (Figure 19). This decreased rectal temperature was at least 2°C less than the control groups.

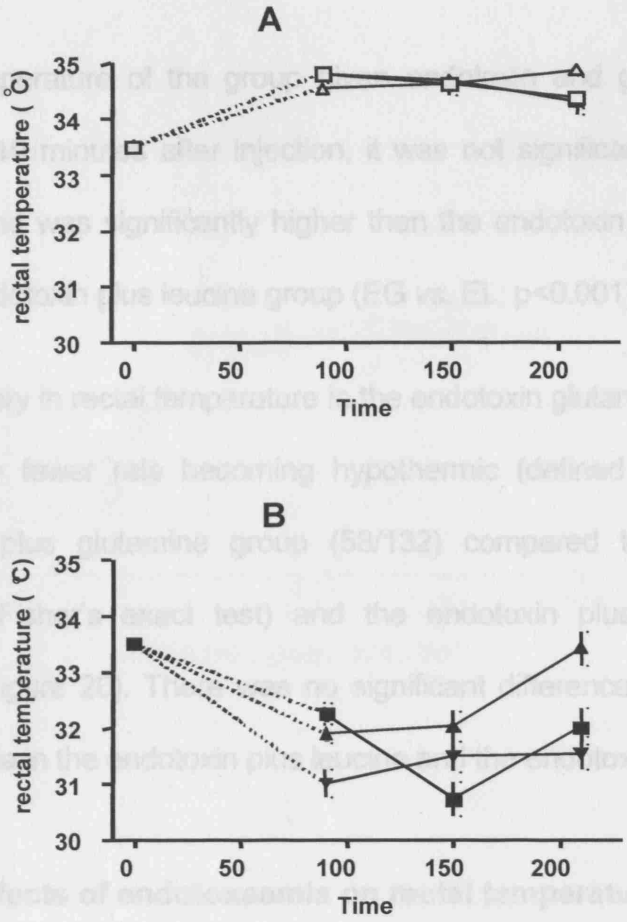


Figure 19 Rectal temperature of rats after injection

A saline [□], saline plus glutamine [△], saline plus leucine [▽]

B saline plus endotoxin [■], saline plus endotoxin plus glutamine [▲], saline plus endotoxin plus leucine [▼]

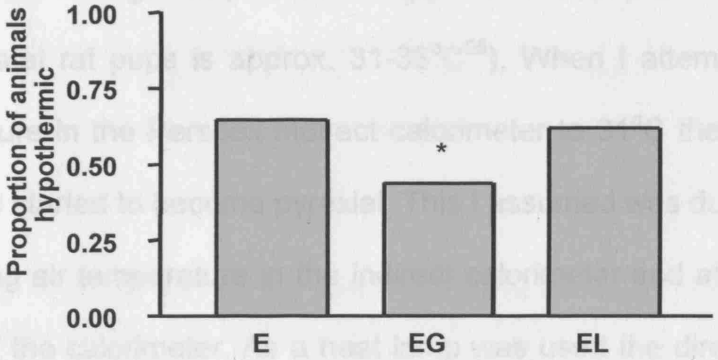


Figure 20 Proportion of animals hypothermic

Proportion of rats given endotoxin alone [E], endotoxin plus glutamine [EG] or endotoxin plus leucine [EL] being hypothermic (<32°C) between 90 and 210 minutes. * $p<0.006$ *vs.* E and L groups, Fisher's exact test.

Rectal temperature of the group given endotoxin and glutamine (EG) recovered. Thus, at 210 minutes after injection, it was not significantly different from controls ($p>0.05$) and was significantly higher than the endotoxin group (EG *vs.* E; $p<0.01$) and the endotoxin plus leucine group (EG *vs.* EL; $p<0.001$) (Figure 19B).

This recovery in rectal temperature in the endotoxin glutamine group (EG) resulted in significantly fewer rats becoming hypothermic (defined as temp < 32°C) in the endotoxin plus glutamine group (58/132) compared to the endotoxin (95/147; $p=0.0007$ Fisher's exact test) and the endotoxin plus leucine groups (74/120; $p=0.006$; Figure 20). There was no significant difference between the incidence of hypothermia in the endotoxin plus leucine and the endotoxin groups.

7.4.1.2 Effects of endotoxaemia on rectal temperatures under heat lamp

In order to assess whether the decreases in rectal temperature were due to reduced huddling of rat pups or due to changes in metabolism I repeated the experiments while maintaining the rats at thermoneutral temperature. After warming of the cage temperature to approx. 28°C (reported thermoneutral range for neonatal rat pups is approx. 31-33°C[95]). When I attempted to increase the temperature in the Perspex indirect calorimeter to 31°C the control rat pups (C) over time started to become pyrexial. This I assumed was due to the difficulties of measuring air temperature in the indirect calorimeter and also due to the closed nature of the calorimeter. As a heat lamp was used the direct effect of the lamp on the rat pups was probably greater than the effect on the calorimeter air

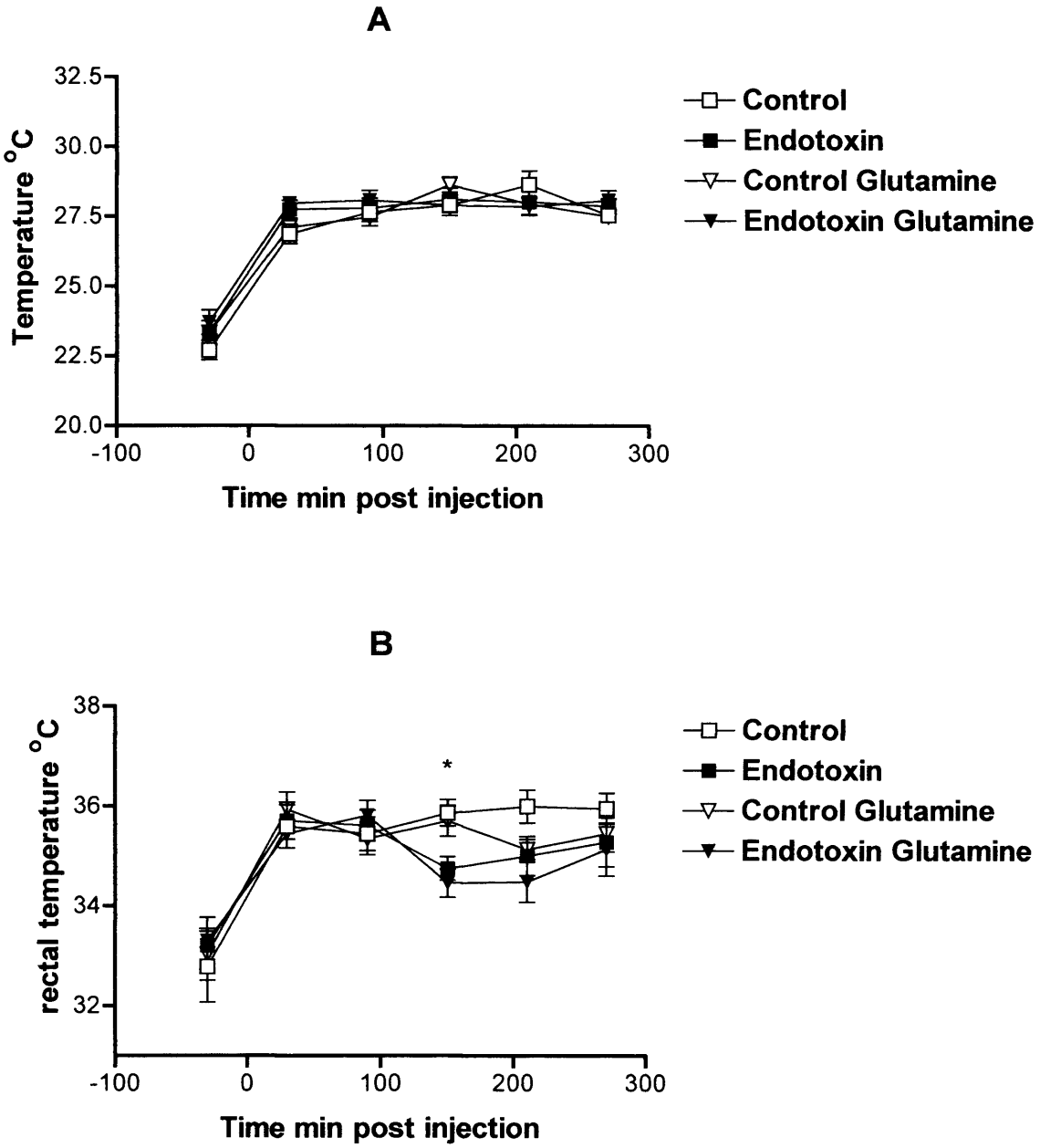


Figure 21 Cage and rectal temperature under heat lamp

A Calorimeter cage temperature under heat lamp *vs.* time post injection

B Rectal temperature of rats under heat lamp *vs.* time post injection
*p<0.05 Control *vs.* Endotoxin and Control Glutamine *vs.* Endotoxin Glutamine

temperature. The calorimeter was therefore heated to a temperature of 28°C, the temperature at which the control animals were thermoneutral.

Throughout the experiments there was no significant difference in the cage temperature of the any of the groups. (Figure 21A)

There was still a slight decrease in rectal temperature of the endotoxin group rats at 150 minutes post injection. This was a much smaller decrease in rectal temperature in the endotoxin groups under the heat lamp (Figure 21B), than in the endotoxin groups not under a heat lamp (Figure 19B). This difference, of rats under the heat lamp, was still a significant difference at 150min (One way ANOVA $p=0.0025$) between control (C) and endotoxin (E) groups (Post test Newman-Keuls multiple comparison test $p<0.05$) and between control glutamine (CG) and endotoxin glutamine (EG) groups (Post test Newman-Keuls multiple comparison test $p<0.05$), despite a cage temperature of 28°C. There was however no significant difference between E and EG groups ($p>0.05$).

7.4.2 Oxygen consumption of control groups

The indirect calorimeter was at ambient room temperature. Before injection, average oxygen consumption (VO_2) of all groups of animals was 1062±43 ml/kg/min. In the control group (C), VO_2 dropped slowly during the course of the experiment, reaching 725±110 ml/kg/min at 210 minutes (Figure 22A). Rats injected with saline plus glutamine (CG) or saline plus leucine (CL) had a higher VO_2 than saline injected rats at all time points after injections (Figure 22A). There

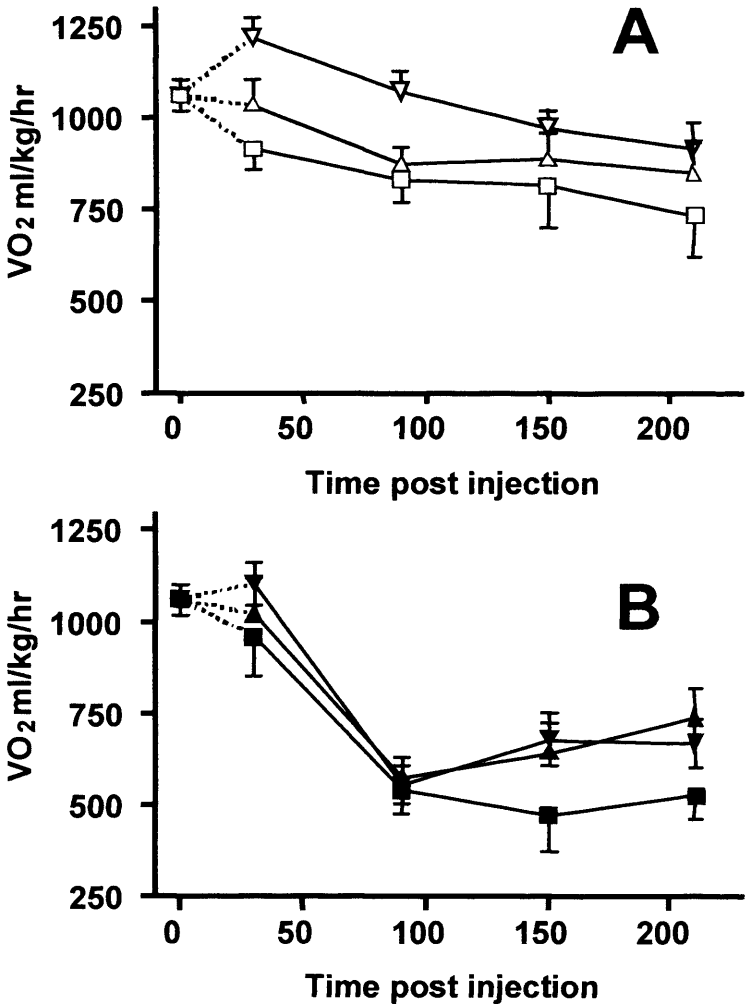


Figure 22 Oxygen consumption (VO_2) of rats
Injected with:

A saline [□], saline plus glutamine [△], saline plus leucine [▽]

B saline plus endotoxin [■], saline plus endotoxin plus glutamine [▲], saline plus endotoxin plus leucine [▼].

was a gradual decline in all of the control groups (C, CG, CL) through the time course of the experiments though this was not statistically significant. There was no significant difference between the control group (C) and the control glutamine (CG) group at any of the time points during the experiments. There was however a significant increase in the oxygen consumption of the control leucine group (CL) compared with control group (C) ($p<0.01$).

7.4.3 Endotoxin effects on oxygen consumption

Endotoxin injected rats had a lower oxygen consumption than control rats (Figure 22B), so that at the end of the experiment there was a significant difference (VO_2 from 150-210minutes: C 671±45; E 429±36, $p<0.0004$ n=8 paired t-test). VO_2 of rats injected with endotoxin and either glutamine or leucine, however, was increased compared to rats injected with endotoxin alone (Figure 22B). When the data were averaged between 90 and 210 minutes, these trends became more apparent (Figure 23). Oxygen consumption of control rats injected with glutamine (CG) or leucine (CL) was greater than control (C) rats (significantly so in the leucine group; $p<0.01$). Whereas that of endotoxin (E) injected rats were significantly decreased compared to all three control groups ($p<0.05$ *vs.* control (C), $p<0.001$ *vs.* control plus glutamine(CG) and control plus leucine (CL)).

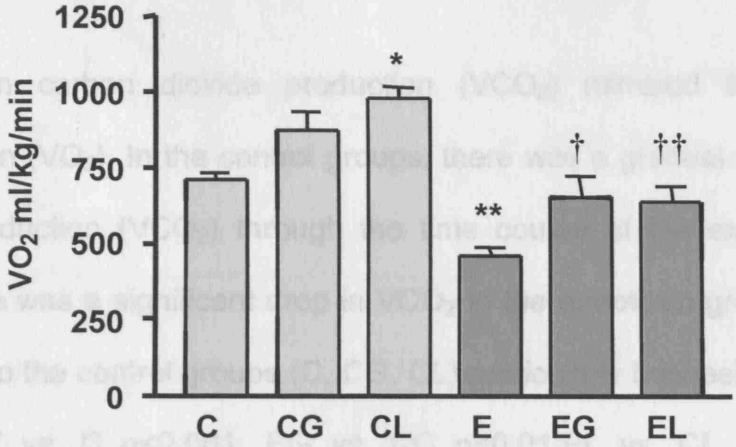


Figure 23 Oxygen consumption averaged between 90-210 minutes
VO2 averaged from 90-210 minutes post injection with saline [C], saline plus glutamine [CG], saline plus leucine [CL], saline plus endotoxin [E], saline plus endotoxin plus glutamine [EG] or saline plus endotoxin plus leucine [EL].
* $p<0.01$ *vs.* C, ** $p<0.05$ *vs.* C, $p<0.001$ *vs.* CG and CL,
† $p<0.05$ *vs.* E and CG, $p=<0.001$ *vs.* CL, †† $p<0.001$ *vs.* CL,
One-way ANOVA with Newman-Keuls post-hoc test.

7.4.4 Glutamine effects on oxygen consumption in endotoxaemia

Between 90-210min post injection, endotoxin rats injected with leucine or with glutamine had a higher VO_2 than rats injected with endotoxin alone, although this difference was only significant in the glutamine injected group ($p<0.05$) (Figure 23). The VO_2 of endotoxin rats injected with leucine or glutamine was not significantly different from that of control (C) animals (although it was still significantly lower than animals injected with saline plus leucine (CL) or saline plus glutamine (CG)).

7.4.5 Carbon Dioxide production

Changes in carbon dioxide production (VCO_2) mirrored those in oxygen consumption (VO_2). In the control groups, there was a gradual decline in carbon dioxide production (VCO_2) through the time course of the experiment (Figure 24A). There was a significant drop in VCO_2 in the endotoxin groups (E, EG, EL) compared to the control groups (C, CG, CL) particularly between 90-210min post injection (E *vs.* C $p<0.001$; EG *vs.* CG $p<0.01$;EL *vs.* CL $p<0.001$) (Figure 24B,C). There was an increase in the carbon dioxide production in the endotoxin glutamine group (EG) but this was not statistically significant compared to endotoxin group (E). There was also an increase in VCO_2 of the endotoxin leucine group (EL) between 90-150min, however this improvement tailed off thereafter.

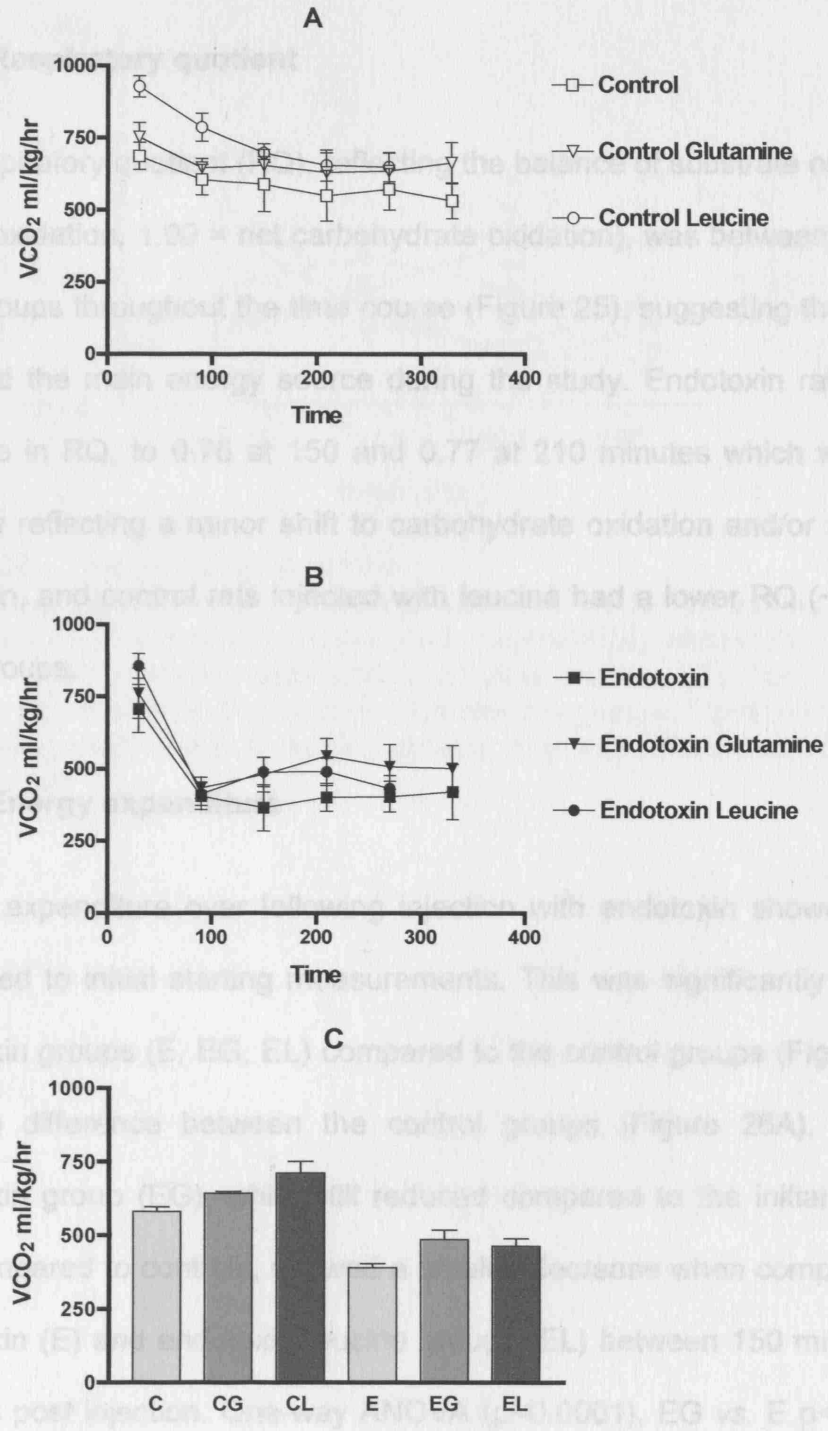


Figure 24 Carbon dioxide production (VCO_2)

A Control groups VCO_2 *vs.* time

B Endotoxin groups VCO_2 *vs.* time

C VCO_2 at 90-210 minutes

7.4.6 Respiratory quotient

The respiratory quotient (RQ), reflecting the balance of substrate oxidation (0.71= net fat oxidation, 1.00 = net carbohydrate oxidation), was between 0.78 and 0.71 in all groups throughout the time course (Figure 25), suggesting that fat oxidation provided the main energy source during the study. Endotoxin rats had a slight increase in RQ, to 0.76 at 150 and 0.77 at 210 minutes which was significant, possibly reflecting a minor shift to carbohydrate oxidation and/or inhibition of fat oxidation, and control rats injected with leucine had a lower RQ (~0.71) than the other groups.

7.4.7 Energy expenditure

Energy expenditure over following injection with endotoxin showed a decrease compared to initial starting measurements. This was significantly reduced in all endotoxin groups (E, EG, EL) compared to the control groups (Figure 26). There was no difference between the control groups (Figure 26A). However, the endotoxin group (EG), while still reduced compared to the initial measurement and compared to controls, showed a smaller decrease when compared with both endotoxin (E) and endotoxin leucine groups (EL) between 150 minutes and 330 minutes post injection. One-way ANOVA ($p<0.0001$), EG *vs.* E $p<0.001$, EG *vs.* EL $p<0.001$ (Figure 26B). There was no difference between the endotoxin and endotoxin leucine groups (E *vs.* EL $p>0.05$).

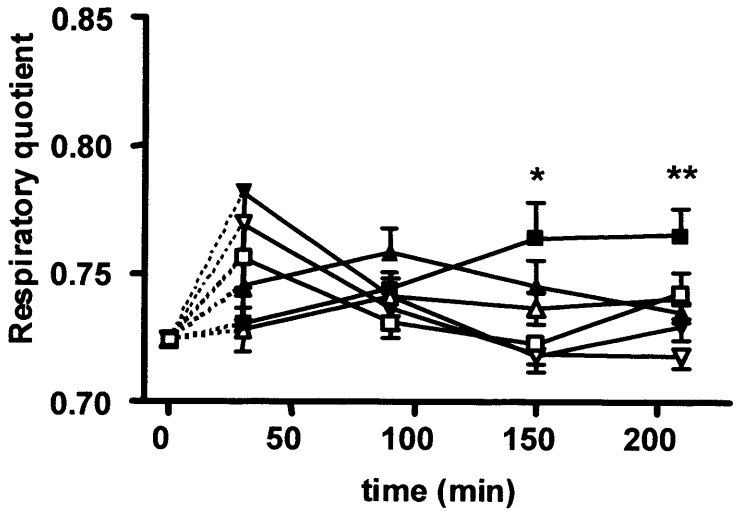


Figure 25 Respiratory quotient
Respiratory quotient after injection of rats with saline [□], saline plus glutamine [□], saline plus leucine [□], saline plus endotoxin [■], saline plus endotoxin plus glutamine [▲] or saline plus endotoxin plus leucine [▼]. *$p<0.01$ *vs.* control, control plus leucine and endotoxin plus leucine groups; **$p<0.05$ *vs.* control plus leucine and endotoxin plus leucine groups, one-way ANOVA with Newman-Keuls post-hoc test.

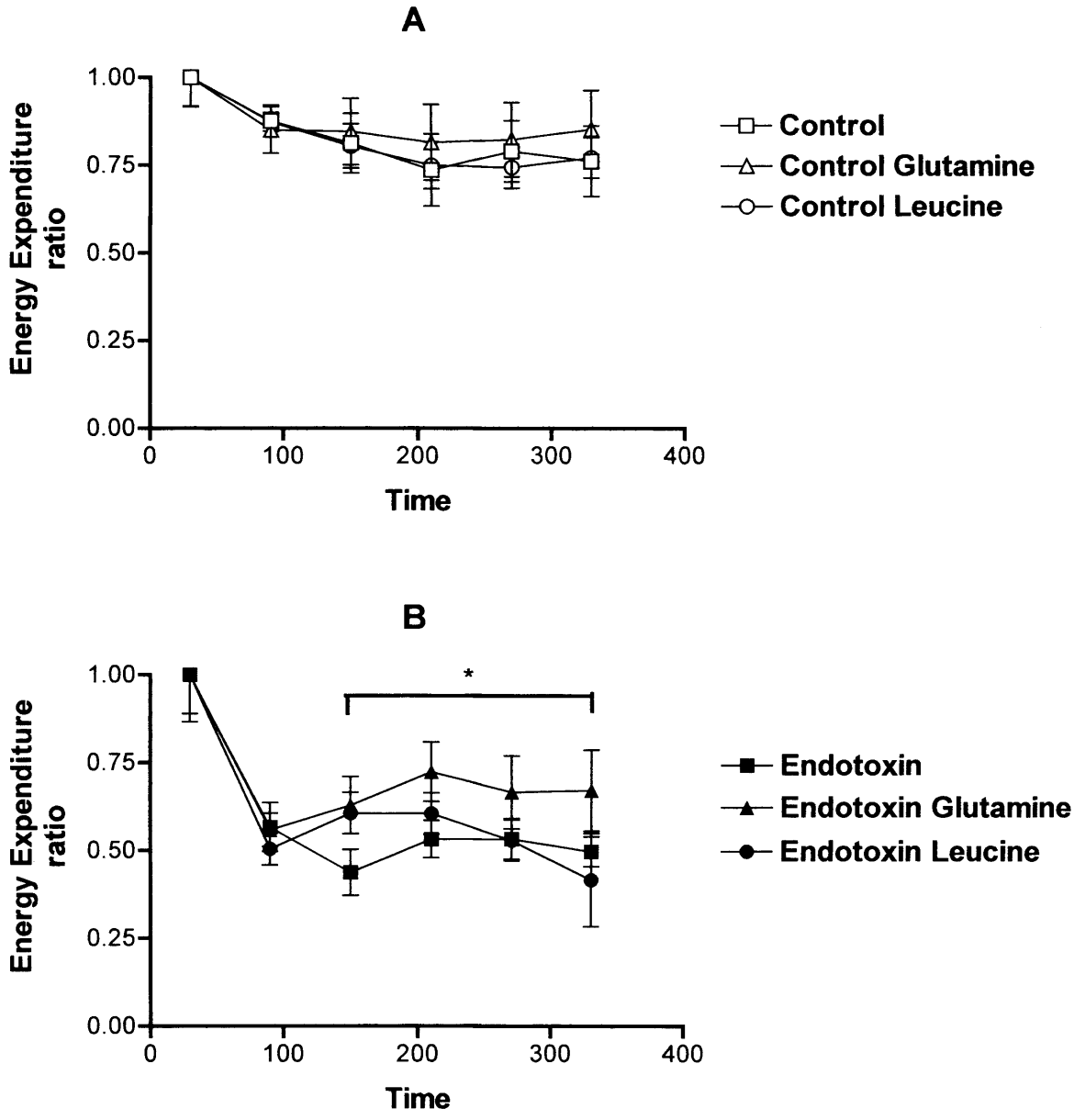


Figure 26 Energy expenditure ratio
Ratio of energy expenditure compared to initial measurement
A Control, Control Glutamine, Control Leucine
B Endotoxin, Endotoxin Glutamine, Endotoxin Leucine
*p<0.001 E *vs.* EG, EL *vs.* EG between 150-330 min
*p>0.05 E *vs.* EL between 150-330 min
One-way ANOVA, Newman-Keuls Post test

7.4.8 Indirect Calorimetry under heat lamp at 28°C

As expected with the increase in cage temperature to 28°C there was a subsequent decrease in the oxygen consumption (VO_2) of all groups compared to initial oxygen consumption measurements at room temperature (time –30min) (Figure 27). There was no significant difference between any of the groups at any of the time points under these conditions (One-way ANOVA $p>0.05$).

There was also reduced energy expenditure with the increased cage temperature. However, there was no significant difference between any of the groups at any of these time conditions (One-way ANOVA $p>0.05$)(Figure 28).

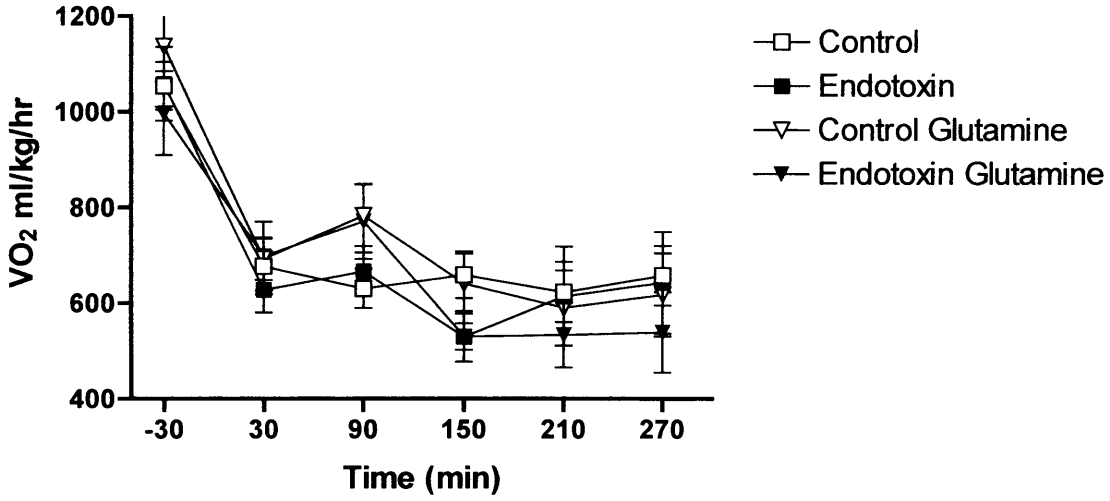


Figure 27 Oxygen consumption in indirect calorimeter under heat lamp Cage temperature at 28°C
Time –30 min Calorimeter at room temperature
Time 0 – 270 min Calorimeter under heat lamp at 28°C

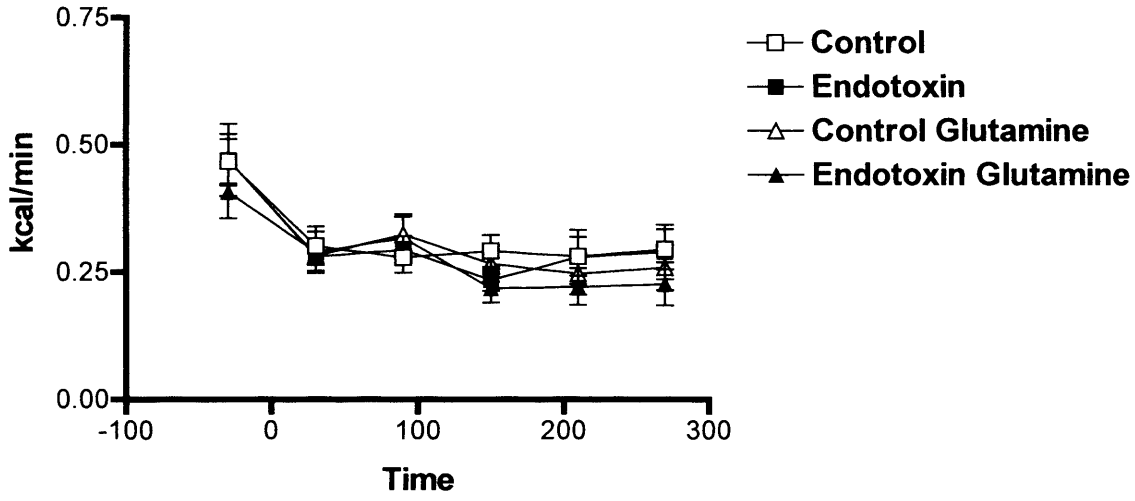


Figure 28 Energy expenditure in indirect calorimeter under heat lamp
Time –30 min Calorimeter at room temperature
Time 0 – 270 min Calorimeter under heat lamp at 28°C

7.4.9 Relationship between temperature and oxygen consumption

The oxygen consumption of the control animals compared to cage temperature showed a strong inverse linear relationship with oxygen consumption (VO_2) at increased cage temperatures within the range of 22-29°C (r^2=0.81, p<0.0001, n=48). This suggests that as the temperature increases towards thermoneutrality, less oxygen consumption is needed for heat production and therefore less total oxygen consumption (VO_2) occurs. (Figure 29)

Under conditions when the cage was not heated, there was a strong negative correlation between VO_2 and rectal temperature in the animals given endotoxin (Figure 30A) (r^2=0.43, p<0.0001), endotoxin plus glutamine (Figure 30B) (r^2=0.51, p<0.0001), and endotoxin plus leucine (Figure 30C) (r^2=0.70, p<0.0001). The range of measured rectal temperatures in the control groups was

small and therefore was insufficient to evaluate whether there was a relationship between temperature and VO_2.

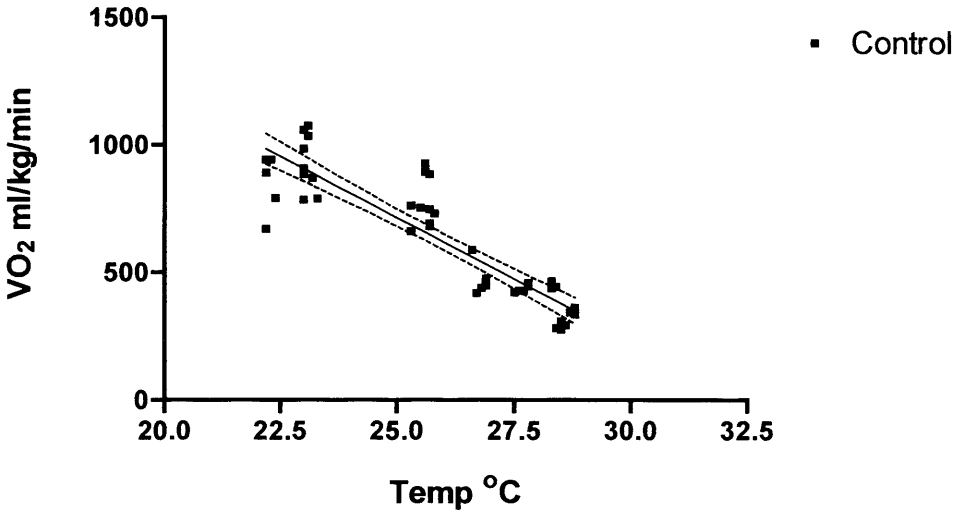


Figure 29 Oxygen consumption of control animals *vs.* cage temperature
Linear regression r2=0.81, p<0.0001, n=48

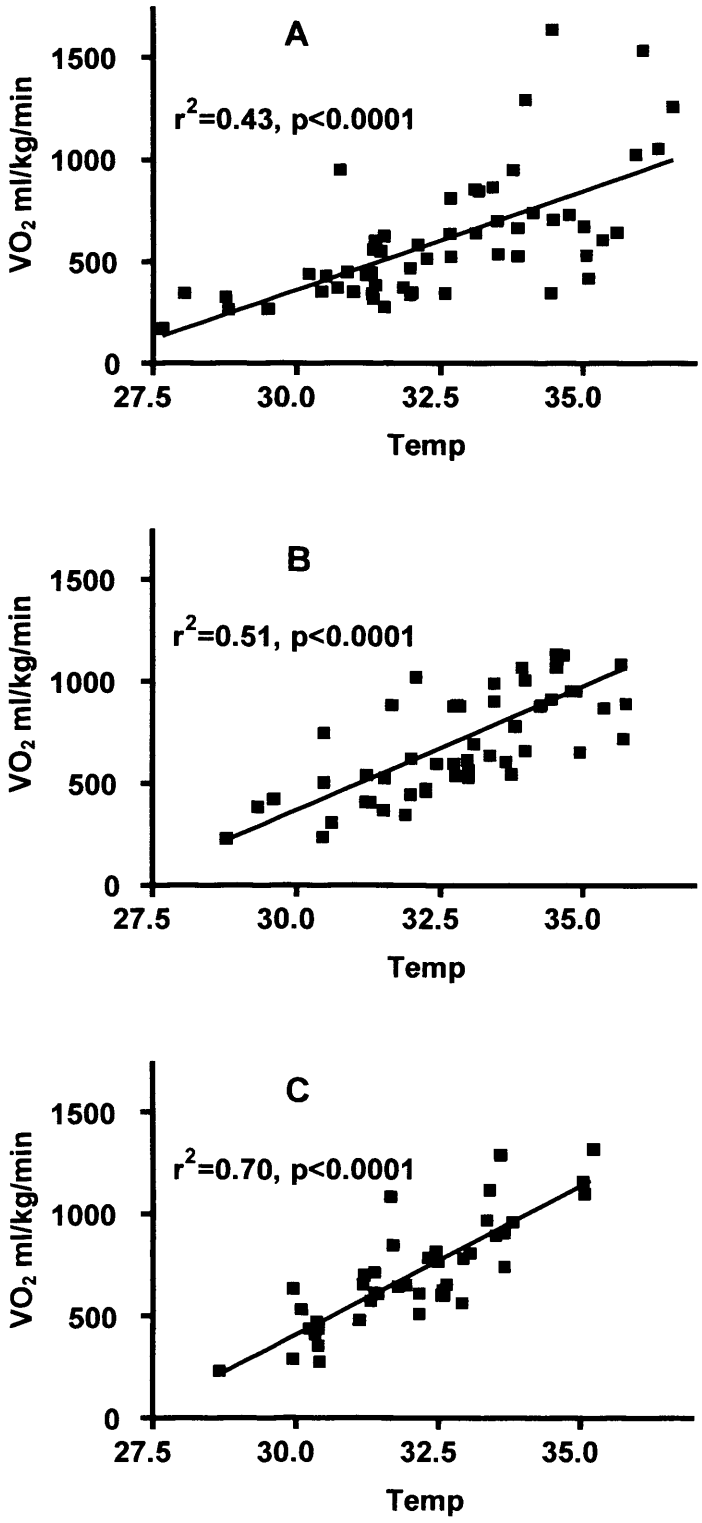


Figure 30 Oxygen consumption *vs.* rectal temperature

A	Endotoxin group	r^2=0.43, p<0.0001
B	Endotoxin Glutamine group	r^2=0.51, p<0.0001
C	Endotoxin Leucine group	r^2=0.70, p<0.0001

7.5 Discussion

7.5.1 Hypometabolism

Ex vivo studies on tissues isolated from our suckling animal model of peritoneal sepsis have indicated that hepatocyte oxidative metabolism[75] and cardiac mitochondrial function are inhibited[96]. Here, I have shown for the first time in a neonatal model of endotoxaemia, that this is accompanied by a profound whole body hypometabolism, which may contribute to the hypothermia observed in these rats. The oxygen consumption of saline injected animals (C) also drops during the course of the experiment; this is probably due to repeated handling and measurement of rectal temperature of the animals, but both VO_2 and temperature of endotoxaemic rats (E) were clearly lower than the control animals (C). The low initial rectal temperatures of the suckling rats is in keeping with the findings of Bertin et al., who showed a steady increase in rectal temperature of rats during development and a temperature of 33.5°C in 11 day old rats[94]. These observed effects of endotoxin on VO_2 and temperature are in keeping with those of DeRijk et al. in adult rats[24,25,26] although other authors have shown increases in both VO_2 and body temperature in adult rat endotoxaemia[97]; these responses may vary with both dose and serotype of endotoxin[98]. Studies in infants and children have not demonstrated a hypo- or hyper-metabolic phase of sepsis[18,99,21,19]; however there may be a hypometabolism which occurs early in sepsis and has consequently not been observed in these studies.

The respiratory quotient of these rats was low; around 0.75. This is in keeping with findings of other authors[94] and reflects the reliance of suckling rats on fatty acids as an oxidative fuel[100,101]. The respiratory quotient was not corrected for urinary nitrogen output, so does include a component of protein oxidation. However, as mixed protein oxidation has a respiratory quotient of around 0.835[102], protein oxidation does not appear to be high in any of the experimental groups. Rats at this age feed more or less continually and so in order to avoid starvation were replaced with their dams for 30 minutes every hour throughout the experiment. At sacrifice and laparotomy, the rat pup's stomachs were distended with milk in all the experimental groups, supporting the lack of starvation during the experiment. However, endotoxaemia is known to cause paralytic ileus in rats[103] so it is possible that endotoxic rats had impaired absorption. There was a slight increase in respiratory quotient in endotoxic rats at the later time points, possibly reflecting a minor shift in the balance of substrate oxidation.

The ambient temperature throughout the experiments was around 23°C. This is well below the range of thermoneutrality for rats of this age (~33-34°C)[104,105,106], and in order to maintain body temperature, rats respond both by facultative thermogenesis[94,95], as in the human neonate[24,25,26,107], and by behavioural adaptations i.e. huddling[108]. Allowing rats to suckle with their dams and measurement of 5 rats together in a metabolic cage minimised uncontrolled heat loss in these animals. Although endotoxaemia could be causing hypothermia in these animals by promoting vasodilatation, decreasing huddling behaviour and

increasing heat losses, the strong relationship between rectal temperature and VO_2 in endotoxic animals would suggest that thermogenesis itself is involved. If the decrease in rectal temperature was due to increased vasodilatation and decreased huddling behaviour alone, then it would seem reasonable to speculate that there would be expected to be an increase in VO_2 to compensate for the increased heat loss. Cytokines, prostaglandins, leukotrienes and the vagus nerve have all been implicated in the reduced thermogenesis observed in endotoxaemia[24,25,26,109,110] but the physiological basis is still uncertain. The reduction of energy expenditure shown here in endotoxaemia also supports this, with the reduced energy expenditure also leading to hypothermia.

In neonatal hepatocytes, it has been shown that glutamine and its dipeptides reversed the inhibition of oxidative metabolism caused by mediators by providing a substrate for glutathione synthesis[74]. Subsequently, it was shown that incubation of hepatocytes from endotoxic rats with glutamine restored their mitochondrial function and reversed ultrastructural changes[75]. In the current study, I wanted to determine the effect of intraperitoneal glutamine injection on the *in vivo* metabolism of endotoxic suckling rats. I also injected rats with leucine as a control to determine whether effects I observed were specific to glutamine.

I showed that glutamine does substantially attenuate the decrease in metabolism seen in endotoxaemia. This attenuation of the effect of endotoxaemia by glutamine represents a significant improvement in the metabolism of the experimental animals that could maintain homeostasis within physiological ranges of the whole body metabolism kinetics. This could in turn prevent organ

failure that is the first step towards multi-organ failure and increased mortality. This is particularly relevant to neonatal patients who have a significant risk of multi-organ failure and a mortality rate that can approach 15-20% in some series[111].

Nutrient-induced thermogenesis (NIT) is an increase in energy expenditure that occurs upon feeding, whether orally or intravenously[112]. Protein/amino acids have by far the greatest thermogenic effect. Although NIT may, in part, be due to effects on the sympathetic nervous system[113,114], the finding that amino acid and glucose-induced NIT persists in tetraplegic patients suggests that other mechanisms, such as the energy dissipated directly by oxidation or non-oxidative disposal of fuels, are also operative[115,116,112]. In my study, glutamine and leucine administration to control rats led to increased VO_2 of control animals, which is probably due to NIT, and supports the assumption that leucine and glutamine are absorbed from the peritoneum[117,118]. This is also supported by the findings and results in Chapter 3. Although it is not known which amino acids have a more thermogenic effect, the finding that leucine had a larger effect on VO_2 than glutamine, in the control groups, suggests that leucine is a more thermogenic amino acid than glutamine. Leucine has previously been suggested to be a strongly thermogenic amino acid[119,120,121]. Although the thermogenic effects of glutamine have not been reported, and NIT is not simply due to oxidative usage of amino acids as fuels, it is noteworthy that leucine has a metabolizable heat of combustion approximately twice that of glutamine[102]. I therefore used leucine as

a control amino acid as it seemed reasonable to assume that it would cause at least as much, and possibly even greater, NIT as glutamine.

Endotoxic rat pups also injected with glutamine or leucine had an increased VO_2 from about 90 minutes after injection compared to endotoxic rats, suggesting that NIT is also occurring in endotoxic rats, as in septic humans[122]. However, unlike control animals, in endotoxic animals, glutamine had a slightly greater thermogenic effect than leucine. This could be related to increased oxidation of glutamine during endotoxaemia[42,28,123,124,125,126,127,128]. Although both glutamine and leucine caused an increase in VO_2 of endotoxic animals the effect of glutamine was greater than that of leucine. However, only glutamine appeared to have an effect on energy expenditure and on rectal temperature and was effective in reducing the number of episodes of hypothermia compared to endotoxaemic rats. It is not known whether glutamine directly or indirectly affects thermoregulation, although a recent study suggested that direct effects of glutamine on cerebral thermoregulatory centres are unlikely[129,8]. Glutamine could act indirectly by altering circulating levels of cytokines, or via effects on prostaglandin or leukotriene metabolism[24,25,26,109,110], but the physiological basis for the action of glutamine on body temperature during endotoxaemia requires further work.

Both leucine and glutamine appear to cause an increased VO_2 in control and endotoxaemic animals, whereas only glutamine was able to reduce both the number of episodes of hypothermia and the endotoxaemia score. It therefore appears that the beneficial effect of glutamine in this model is at least partly

independent from an effect on NIT. Glutamine also showed a beneficial effect on the energy expenditure in endotoxaemia that was not seen with leucine in endotoxaemia. Glutamine in TPN reduces mortality to a lethal bacterial challenge[130] and has been shown to have various beneficial effects in clinical trials, such as reduced nitrogen loss[42], prevention of mucosal atrophy[61] and preservation of mucosal structure[62,63]. Our results suggest that glutamine may additionally help prevent sepsis-related hypometabolism and hypothermia and appears to be specific to glutamine.

8 Plasma Inflammatory markers

8.1 Introduction

Cytokines are intrinsically involved in the effect of sepsis or endotoxaemia in humans and animals. They have effects both locally on neighboring cells as well as on other organs via the blood stream. Cytokine plasma concentrations can therefore give an indication of the effect of sepsis or endotoxaemia on the animal. Cytokines are released by inflammatory cells in response to a stimuli e.g. endotoxin. Cytokines can be either pro-inflammatory e.g. Tumour necrosis factor alpha (TNFα) or ant-inflammatory e.g. Interleukin 10 (IL10). Cytokines are released as a cascade stimulating different effects on different cells.

8.2 Hypothesis

Hypothesis that glutamine may be modulating the response to endotoxaemia via the inflammatory response and therefore there may be changes in concentrations of cytokines reducing the septic inflammatory response.

8.3 Methods

8.3.1 Tumour necrosis factor alpha (TNF-α) measurement

A rat specific TNF-α Enzyme Linked Immuno-Sorbent Assay (ELISA, Biosource International) was used to determine TNF-α levels in plasma. This is a solid phase sandwich ELISA. An antibody specific for rat TNF-α is coated onto the wells of the microtiter strips. Samples, including standards of known rat TNF-α content, control specimens, and unknowns were pipetted into these wells, followed by the addition of a biotinylated secondary antibody. During the first incubation, the rat TNF-α antigen bound simultaneously to the immobilised (capture) antibody on one site, and to the solution phase biotinylated antibody on a second site. After removal of excess second antibody, Streptavidin-Peroxidase (enzyme) is added. This binds to the biotinylated antibody to complete the four-member sandwich. After a second incubation and washing to remove all the unbound enzyme, a substrate solution, stabilised chromagen (tetramethylbenzidine) is added, which is acted upon by the bound enzyme to produce colour. The intensity of this coloured product is directly proportional to the concentration of rat TNF-α present in the original specimen. The absorbance of each well is measured at 450nm and compared to standard solutions of rat TNF-α to calculate the concentration of TNF-α in plasma.

8.3.2 Interleukin 10 (IL-10) measurement

Plasma IL-10 was measured by a rat IL-10 ELISA (BioSource International, Inc.). This is a four layer sandwich similar to the TNF-α ELISA described above. Again the absorbance of each well was measured at 450nm and compared to standard solutions of rat IL-10 to calculate the concentration of IL-10 in plasma. The manufacturers state no cross-reactivity with IL-1β, IL-4, IFN-γ, MIP-2, or TNF-α and a minimum detectable amount of rat IL-10 of <5pg/ml.

8.3.3 Nitric Oxide (NO) Measurement

Plasma nitrate (NO_3^-) and nitrite (NO_2^-) concentrations were measured as an indicative measure of nitric oxide concentrations. Nitrate and nitrite are the breakdown products of nitric oxide. As nitric oxide has a very short half-life it is necessary to measure the concentrations of breakdown products nitrate and nitrite and extrapolate from these to the relative concentrations of nitric oxide (NO). The principle of the assay is that nitrate is reduced to nitrite by incubation with nitrate reductase, followed by reaction of total nitrite (nitrate plus nitrite) with a fluorescent derivatising agent[131].

Initially standard curves are measured by testing samples of known concentration of nitrate and nitrite between 200μM and 10μM as well as zero.

Plasma/samples (5μl) are incubated with 20μl 68.75μM NADPH and 20μl 825U/l nitrate reductase at room temperature for 3 hours. At 3 hours 63μl 480μM N-methyl-4-hydrazino-7-nitrobenzofurazan (MNBDH) and 67.2μl 20% H_3PO_4 are added and the sample is further incubated for 30 minutes.

The samples are then analysed via high performance liquid chromatography (HPLC). The flow rate was 0.62ml/min, and a gradient of 50mM ammonium acetate (pH 7.5) against acetonitrile was run on a Hypersil 5mODS column with a Phenomenex Security Guard ODS cartridge. Initial conditions were: 45% buffer and 55% acetonitrile, for 1.5 minutes, followed by a gradient to 100% acetonitrile 8.5min, which was continued for 4 minutes. A fluorescence detector (JASCO FP-1520) monitored the column effluent at an excitation wavelength of 468nm and an emission wavelength of 537nm. Concentrations are calculated by comparison with the standard graphs.

8.3.4 Statistical Analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical comparison of multiple groups was performed by One-way ANOVA with Newman-Keuls Multiple Comparison post-test. Prism 3.02 and Instat 3.05 (both GraphPad Software, San Diego California USA) were used for statistical comparisons.

8.4 Results

8.4.1 Plasma TNF-α concentration

In the control and control glutamine groups only minimal concentrations of TNF-α were detectable at either 2 hours (C=26.6±6.5 pg/ml, CG=79.5±17.2 pg/ml) or 6 hours (C=32.1±26.8 pg/ml, CG=20.1±2.3 pg/ml) post injection. However, in the endotoxin (E) and endotoxin glutamine groups, there was a massive rise in TNF-α levels at 2 hours post injection (E=2247±43pg/ml, EG=1991±91pg/ml) (Figure 31A). By 6 hours post injection (E=799±193pg/ml, EG=219±75pg/ml) (Figure 31B), while still markedly raised, the concentrations were much reduced compared to the concentrations seen at 2 hours post injection. However the endotoxin glutamine group (EG) was significantly reduced compared to the endotoxin group (E) at both 2 hours ($p<0.01$) and at 6 hours ($p<0.01$). At 6 hours post injection the endotoxin glutamine (EG) TNF-α plasma levels are less than 30% of the concentrations measured in the endotoxin group (E).

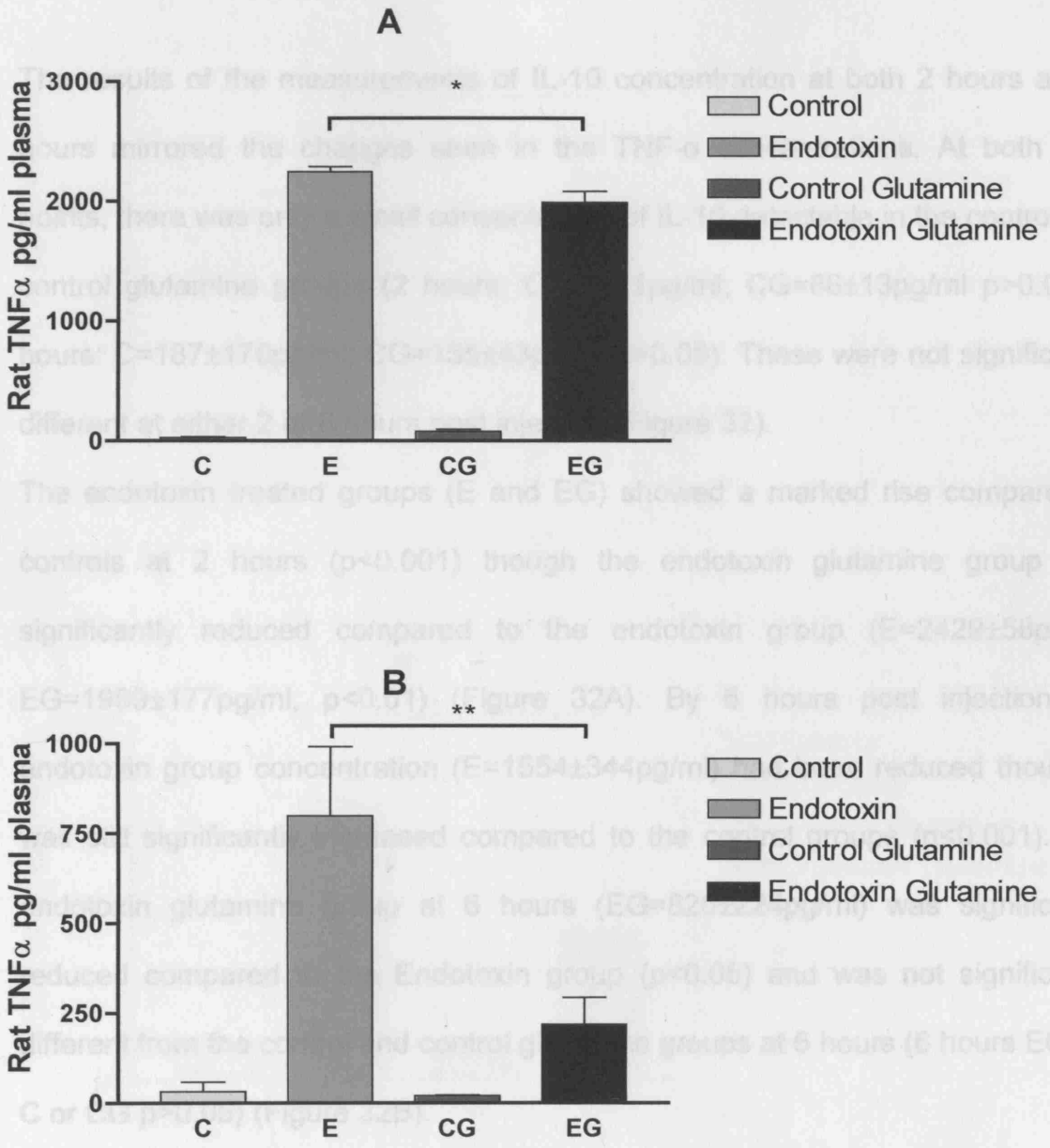


Figure 31 Plasma Tumour Necrosis Factor-α (TNF-α) concentrations
A 2 hours post injection
*p<0.01 Endotoxin *vs.* Endotoxin Glutamine (n=10)
B 6 hours post injection
**p<0.01 Endotoxin *vs.* Endotoxin Glutamine (n=7)

8.4.2 Plasma Interleukin-10 concentration

The results of the measurements of IL-10 concentration at both 2 hours and 6 hours mirrored the changes seen in the TNF-α concentrations. At both time points, there was only a small concentration of IL-10 detectable in the control and control glutamine groups (2 hours: C=55±21pg/ml, CG=88±13pg/ml $p>0.05$; 6 hours: C=187±170pg/ml, CG=155±43pg/ml $p>0.05$). These were not significantly different at either 2 or 6 hours post injection (Figure 32).

The endotoxin treated groups (E and EG) showed a marked rise compared to controls at 2 hours ($p<0.001$) though the endotoxin glutamine group was significantly reduced compared to the endotoxin group (E=2429±58pg/ml, EG=1989±177pg/ml, $p<0.01$) (Figure 32A). By 6 hours post injection the endotoxin group concentration (E=1554±344pg/ml) had been reduced though it was still significantly increased compared to the control groups ($p<0.001$). The endotoxin glutamine group at 6 hours (EG=820±224pg/ml) was significantly reduced compared to the Endotoxin group ($p<0.05$) and was not significantly different from the control and control glutamine groups at 6 hours (6 hours EG *vs.* C or CG $p>0.05$) (Figure 32B).

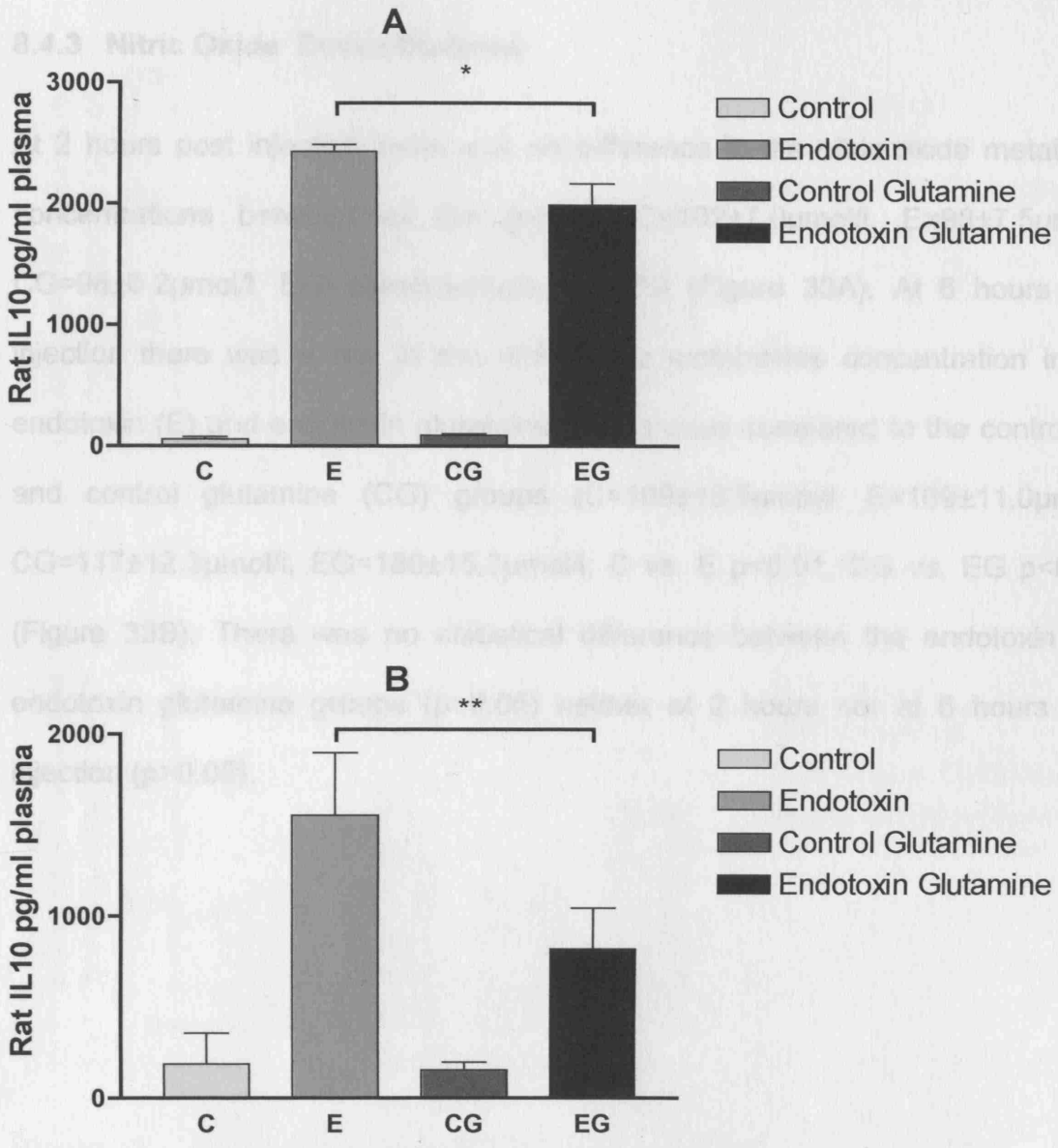


Figure 32 Plasma Interleukin-10 concentrations
A 2 hours post injection
*p<0.01 Endotoxin *vs.* Endotoxin Glutamine (n=10)
B 6 hours post injection
**p<0.05 Endotoxin *vs.* Endotoxin Glutamine (n=7)

8.4.3 Nitric Oxide Concentrations

At 2 hours post injection there was no difference in the nitric oxide metabolite concentrations between all the groups (C=102±7.0μmol/l, E=98±7.5μmol/l, CG=98±6.2μmol/l, EG=104±5.9μmol/l, p=0.89) (Figure 33A). At 6 hours post injection there was a rise in the nitric oxide metabolites concentration in the endotoxin (E) and endotoxin glutamine (EG) groups compared to the control (C) and control glutamine (CG) groups (C=109±10.5μmol/l, E=169±11.0μmol/l, CG=117±12.3μmol/l, EG=186±15.3μmol/l; C *vs.* E $p<0.01$, CG *vs.* EG $p<0.01$) (Figure 33B). There was no statistical difference between the endotoxin and endotoxin glutamine groups ($p>0.05$) neither at 2 hours nor at 6 hours post injection ($p>0.05$).

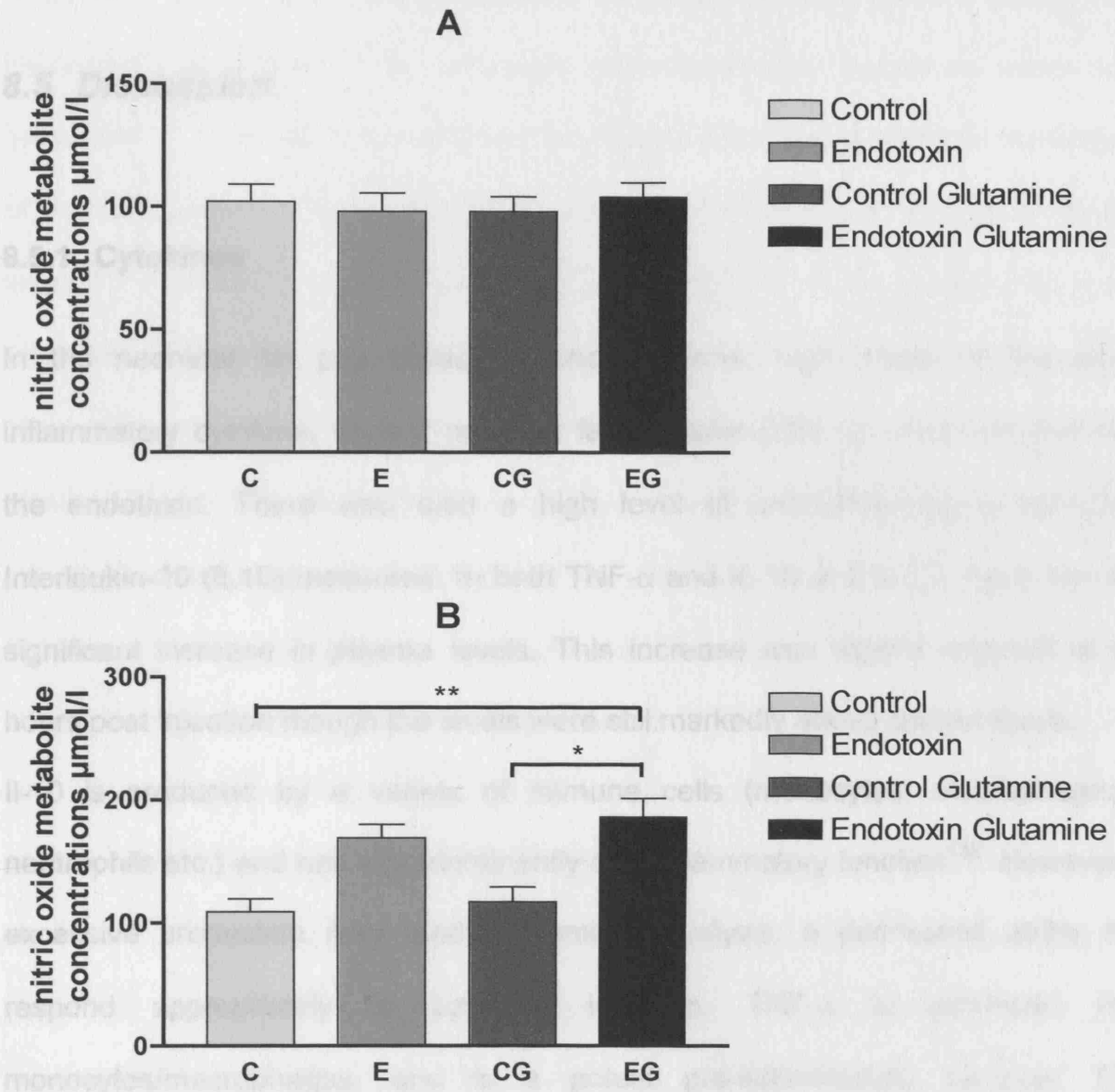


Figure 33 Nitric Oxide metabolite concentrations
A 2 hours post injection
P=0.89 One-way ANOVA
B 6 hours post injection
P=0.0003 One-way ANOVA
*p<0.01 Control Glutamine *vs.* Endotoxin Glutamine (n=7)
**p<0.01 Control *vs.* Endotoxin Glutamine (n=7)

8.5 Discussion

8.5.1 Cytokines

In the neonatal rat pup model of endotoxaemia, high levels of the pro-inflammatory cytokine, tumour necrosis factor alpha (TNF-α), were induced by the endotoxin. There was also a high level of anti-inflammatory cytokine Interleukin-10 (IL10) measured. In both TNF-α and IL-10 at 2 hours there was a significant increase in plasma levels. This increase was slightly reduced at 6 hours post injection though the levels were still markedly above control levels.

Il-10 is produced by a variety of immune cells (monocytes, macrophages, neutrophils etc.) and has a predominantly anti-inflammatory function[132]. However, excessive production may lead to immuno-paralysis, a decreased ability to respond appropriately to bacterial infection. TNF-α . is produced by monocytes/macrophages, and is a potent pro-inflammatory cytokine. Its production is attenuated by IL-10 in an autocrine fashion[133,134]. Several studies, differing in whether *in vivo* or *in vitro*, on the model of sepsis or species of observation, have had inconsistent results as to the effect of glutamine on IL-10 and TNF-α levels. In an adult rat endotoxaemia model, intravenous glutamine significantly decreased plasma TNF-α,. .IL-1β and mortality, and decreased plasma IL-10, although this difference was not significant[135]. In model of gastrostomy-fed endotoxaemic infant rats, glutamine blunted inflammation,

decreasing TNF-α and cytokine-induced neutrophil chemoattractant (CINC; rat homologue of human IL-8), although anti-inflammatory cytokines were not measured[136]. In an adult, parenterally fed, caecal ligation and puncture rat model of sepsis, glutamine supplementation had no effect on TNF-α, IL-6 or IL-1β levels[137]. However in another parenterally-fed adult rat model, alanyl-glutamine dipeptide decreased circulating bacterial numbers in response to an intraperitoneal bacterial challenge, increased hepatic TNF-α and decreased plasma CINC, although plasma TNF-α was unchanged[138]. Glutamine provision in parenteral nutrition of surgical adults did not alter production of IL-2, TNF-α or IL-6 production *ex vivo*, although it did enhance T-cell proliferation[139]. Glutamine-supplemented parenteral nutrition preserved IL-4 and IL-10 expression in lipopolysaccharide-stimulated intestinal cells from mice compared to mice fed parenterally without glutamine supplementation[140]. *In vitro*, glutamine provision increased production of IL-1β and IL-6 by lipopolysaccharide-stimulated human peripheral blood mononuclear cells although IL10, IL-1β or TNF-α production was unaffected[141], decreased IL-6 and IL-8 production, but increased Il-10 production, by human duodenal biopsies stimulated by IL-1β[142]. Peripheral blood mononuclear cells increased proliferation in response to glutamine, and increased production of IL-2 and interferon-γ; whereas IL-1β, TNF-α and IL-6 production were unaffected[143] and glutamine decreased IL-8 production by lipopolysaccharide-stimulated intestinal cells[144]. Cytokine release has also been modulated by glutamine deficiency in human peripheral blood polymorphonuclear

cells and attenuation of the cytokine release has been shown when cultured with glutamine[145,146].

However, it is difficult to interpret the results of these *in vitro* studies in relation to the current study, as the normal plasma concentration of glutamine is 0.75mM, so that *in vivo*, even during endotoxaemia, when glutamine levels fall to 0.3mM, the concentration of glutamine is greater than the unsupplemented *in vitro* studies.

Cytokines and in particular TNF-α have been shown to be critical mediators of sepsis[147]. However, complete blockade of cytokine release including TNF-α leads to significant morbidity and mortality while over expression of TNF-α is also detrimental[148,149]. Therefore attenuation of the cytokine response may prevent the morbidity associated with over expression while allowing the septic inflammatory response to fight infection. Significant clinical effects of glutamine administration may not be found under small to moderate insults with only a corresponding small decrease in plasma glutamine concentration. However severe septic insults may lead to a more significant beneficial effect of glutamine, as larger drops in plasma glutamine concentration may be expected when glutamine utilisation outstrips glutamine release and synthesis. This speculated larger drop in plasma glutamine might lead to significant morbidity unless counteracted by the administration of exogenous glutamine.

More recent studies have shown in human peripheral blood polymorphonuclear cells that glutamine also induces Heat shock protein 72 (HSP70) as well as attenuating the cytokine response[150,151]. Heating of human peripheral blood

polymorphonuclear cells also attenuates the cytokine response while inducing heat shock proteins. Glutamine's mechanism of action on attenuating the cytokine response to a septic stimulus therefore may be related to heat shock protein induction.

8.5.2 Nitric Oxide

The systemic production of nitric oxide is similar to other studies following endotoxin administration to either mice or rats[152,153]; inducible nitric oxide synthase (iNOS) mRNA expression in macrophages may become stimulated rapidly after endotoxin administration[154,155] and it is this isoform that is probably responsible for the increased nitrate plus nitrite production observed here. In contrast to the constitutively-expressed endothelial NOS, which is inhibited by glutamine provision[156], macrophage nitric oxide production through iNOS is stimulated by glutamine[156], possibly by providing an alternative precursor for intracellular arginine[157]. If endothelial NOS is inhibited by glutamine and macrophage nitric oxide production is stimulated by glutamine there will be decreased endothelial nitric oxide production and increased macrophage nitric oxide production. Therefore this may account for the non-significant increase in nitric oxide production caused by provision of glutamine to endotoxic rats observed in the present study, while still possibly having larger local changes in the nitric oxide production than can be measured in the plasma.

9 General Discussion and Conclusions

Glutamine has the potential to be a useful adjunctive treatment in neonatal sepsis as shown by these *in vivo* animal studies. Glutamine has not been a miracle cure in trials done in the adult population as the varying results show. In these experiments I have shown that there is an early decrease in plasma glutamine concentrations in endotoxaemia. This decrease is counteracted by the administration of glutamine. There have been many debates over whether glutamine becomes conditionally essential under conditions of sepsis or stress. Muscle has large stores of glutamine so would need severe conditions to deplete them of glutamine. However, localised glutamine depletion may occur in sepsis, when utilisation of glutamine exceeds the rate of mobilisation of glutamine from muscle, and could be prevented by the administration of glutamine. In the well patient, the body's own mechanisms will be able to correct any localised depletion of glutamine and therefore the administration of glutamine may have a minimal or no discernible effect. However, in the sickest patients it would seem reasonable to hypothesise that there would be greater depletion of glutamine. Assuming this, then the next logical step would be to assume that the early administration of glutamine might prevent the depletion of glutamine in these sickest patients with a more significant effect in clinical response. This could be the reason that the 6 month mortality was reduced in those patients administered glutamine in a clinical trial by Griffiths et al[59]. It is logical to conclude that those that died were the sickest patients and the sickest patients therefore may have

the most to gain by the administration of glutamine. However, studies in premature neonates given routine glutamine supplementation did not show any decrease in infections[77]. There was however no assessment of whether glutamine reduced the morbidity of the severest infections, though there was no discernible difference in mortality.

In these studies that I performed there was a decrease in plasma glutamine concentrations at 2 hours that had nearly resolved by 4 hours. The experimental model that I used, with a single intraperitoneal injection of endotoxin, means that there is only a single insult. However a gram negative bacterial infection would have an on-going release of endotoxin, which may lead to a decrease in plasma glutamine levels that would be more persistent and more prolonged and may not be counteracted by glutamine synthesis or mobilisation. If there is a more prolonged decrease in glutamine this may be having a more significant effect on cells and organs and their metabolism. A recovery in glutamine plasma concentration from administration of glutamine may therefore counteract some of the signs and symptoms of sepsis.

Sepsis is a common cause of morbidity and mortality in neonatal patients, particularly in surgical patients. I have shown that clinical symptoms and signs of endotoxaemia in neonatal rats can be attenuated by the administration of glutamine. Both power and mobility of the neonatal rat pups was decreased in endotoxaemia. The decrease in power and mobility is partially restored by the administration of glutamine in endotoxaemia. Furthermore, hypometabolism seen in this endotoxaemia model of neonatal sepsis can also be partially reversed by

the administration of glutamine. The mechanisms behind the decrease in heat production and oxygen consumption measured by the indirect calorimeter are not clear. I have shown that there were small changes in liver oxygen consumption, however these were not large enough to explain the large changes seen in whole body oxygen consumption. The effects of glutamine administration are probably due to changes in metabolism of many different cell types and organs.

To assess how the endotoxin may be affecting different organs around the body I looked at the cytokine response in each experimental group. There is a reduction in the cytokine response to the endotoxaemia in the group that was administered glutamine (EG). This reduction in the cytokine response may be the explanation for the effects of glutamine in endotoxaemia. If the cytokine response, and thus the inflammatory response, to endotoxin is blunted then there could be less damage due to septic inflammatory response syndrome as the cascade of cytokines is less marked and as a result, there could be a reduction in the oxygen free radical species being released in the animal. This in turn leads to less antioxidant requirement e.g. glutathione. In addition with the administration of glutamine there is the potential for further production of glutathione assuming the availability also of cysteine and glycine.

Glutamine therefore has the potential to be an adjunctive treatment in neonatal sepsis. My work shows that glutamine is probably working in a multi-factorial manner but that the most likely explanation for glutamine's effect is due to

prevention of any local glutamine deficiency when the rate or utilisation outstrips the mobilisation of glutamine from body stores.

A meta-analysis of trials in low-birth weight premature neonates has concluded that routine use of glutamine in this group is unnecessary. However my studies in neonatal rats exposed to a severe endotoxic insult suggest that there may still be a place for administration of glutamine in severely ill septic patients. They are at a greater risk of developing a decrease in glutamine levels to such an extent that glutamine deficiency may cause local and systemic effects on metabolism.

A multicentre clinical trial has been set up, the Surgical Infants Glutamine Nutrition (SIGN) trial, and we await the results of the effect of glutamine administration in the clinical situation of surgical neonatal patients. However a fundamental difficulty in these trials on neonates is the balance between inclusion criteria and numbers eligible for the trial. If only very severely septic neonates are admitted to the trial then the numbers will be small. However if the inclusion criteria are broadened then many of the patients admitted to the trial will not be severely septic and therefore will possibly not show any difference between those administered glutamine and those not.

Though at present the evidence does not show a clinical need in the majority of patients, there is a further argument for administration of glutamine routinely in both enteral and parenteral feeds in neonates. In breast milk there is a high glutamine content. No studies of glutamine administration have shown a detrimental effect. It is therefore reasonable to suggest that in enteral feeds, if breast milk is not available, and in parenteral feeds, one should be supplying

glutamine to try and mimic nature in supplying similar nutrition to that of breast milk. In some areas of the country glutamine is already a component of total parenteral nutrition in neonates with no adverse effects reported.

In conclusion I have shown that in severely ill neonatal rats there is a beneficial effect of glutamine clinically and metabolically. No detrimental effects of glutamine administration have been shown. Therefore I feel that glutamine addition to parenteral nutrition should be considered routinely, despite no beneficial effects having been shown in clinical human neonatal trials, as this will better mimic breast milk nutrition.

Further trials in severely septic neonatal patients may be warranted.

9.1 Addendum: Where next?

The limit to the useful experimental data that can be got from this endotoxaemia model of neonatal sepsis in rat pups has probably been reached. Further clinical human trials may provide useful evidence of the potential benefit of glutamine supplementation to feeds either parenterally or enterally. However assessing the evidence so far in adult and infant/neonatal trials as well as the in vitro and in vivo results suggests that the benefits of glutamine supplementation are mainly likely to be clinically significant in the sickest patients. The sickest patients probably get the most benefit from glutamine as they also drop their glutamine levels the most.

Recent results from the SIGN trial of glutmaine supplementation in surgical infants (reported BAPS Conference 2006) showed that there was a significant reduction in infectious complications in those patients that were on total parenteral nutrition with glutamine supplementation compared to those on total parenteral nutrition without glutamine supplementation. However there was no difference demonstrated in groups on partial parenteral, partial enteral nutrition nor on total enteral nutrition when supplemented with or without glutamine supplementation. One could hypothesise that the group on total parenteral nutrition were most likely to include the sickest patients and therefore the most likely to have the lowest plasma and cellular glutamine levels and therefore the patients in whom glutamine supplementation may raise the glutamine levels in the patient back up to a level at which glutamine is not a rate limiting step in the function of the body.

Clinical trials of glutamine supplementation may therefore be limited as the numbers of neonates and infants who will become significantly unwell to drop their glutamine levels is small and thus it would be hard to recruit a significant number of these patients to a trial. However further work could be done *in vivo* on animal models. The most significant disease process in neonates that may benefit from glutamine supplementation is probably necrotising enterocolitis (NEC). Severe NEC could lead to significant drop in plasma glutamine, particularly as enteral feeding is stopped as part of the treatment of NEC. Therefore a model of NEC may help us to understand the mechanisms and effects of glutamine supplementation. NEC aetiology is not fully understood, but

infection and ischaemia play a role in the development of NEC. Therefore the endotoxaemia model of sepsis in rat pups could be modified to include an element of ischemia. The ischaemia could be induced by reducing the ambient oxygen surrounding the rat pups in a calorimeter chamber. Experiments would need to be done to ascertain the appropriate level of ischemia and endotoxin administration to give a reproducible NEC like effect. If a model can be developed along the above lines there would be scope for experiments to elucidate further the mechanisms and effects of glutamine supplementation.

10 Appendix

10.1 Paediatric Surgical Unit, Institute of Child Health, University College, London

The Institute of Child Health is a leading centre for research into clinical and scientific aspects of childhood disease. It was recently awarded 5* rating. Diverse clinical research in the Paediatric Surgery Unit has led to changes in clinical practice and improvement in outcomes for patients. The metabolic consequences of childhood surgery and parenteral nutrition, including the effects of tissue damage on 'distant' organs such as the liver, brain, kidney and heart, and the effects of anaesthetics and infection on newborns, are being studied.

The Unit is also co-ordinating two large multi-centre randomised controlled trials in neonatal surgery. The first is aimed at determining whether glutamine supplementation in parenteral nutrition reduces the sepsis rate and length of stay in hospital (SIGN trial); the second trial is examining the effect of peritoneal drainage on mortality in neonates who weigh less than 1kg and who have perforated necrotising enterocolitis (NET trial). Gastroenterology research has focused attention on the developmental biology of the gastrointestinal tract and on inflammatory bowel disease.

The surgical unit is a part of the Nutrition unit, ICH. Research in the Nutrition Unit has shown the critical impact of early nutrition on long-term health and development, notably cognition, bone development, the immune system and a major impact on cardiovascular disease including blood pressure, insulin resistance, blood lipids, body fatness and vascular health itself. Novel methods for measuring body composition and energy expenditure in children are also being developed to improve the nutritional management of sick children, and research is also underpinning the development of novel feeding products.

10.2 The work was carried out by myself

With the help of the collaboration detailed below, I carried out all of the work myself.

10.3 Supervision

I met weekly with my supervisors Professor Agostino Pierro and Dr Simon Eaton to discuss my progress. In addition, both were available informally on a day-to-day basis for further help. Their research on total parenteral nutrition, neonatal sepsis and bacterial translocation and metabolism in sepsis enabled them to teach specific research techniques for my project as well as generic research skills. Furthermore, with their encouragement, I presented my work at the British Association of Paediatric Surgeons Conferences at Cambridge, UK 2002 and Estoril, Portugal 2003, at the British Association of Parenteral and Enteral

Nutrition (BAPEN) at Glasgow, UK 2002 at the Royal College of Surgeons of England, Tommy's Research Conference 2002 and at Institute Grand Round Meetings, Laboratory and Departmental Meetings.

10.4 Collaboration

Dr Giorgio Stefanutti and Dr Simon Eaton, Institute of Child Health, London, measured the nitrate and nitrite levels of my experimental samples.

10.5 Prize

Winner of Peter Paul Rickham Prize for best presentation at British Association of Paediatric Surgeons Conference 2002, Cambridge, UK.

10.6 Abstracts

10.6.1 BAPS Conference 2002 Cambridge, UK

Body temperature and heat production in suckling rat endotoxaemia: beneficial effects of glutamine.

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BACKGROUND/PURPOSE: Sepsis is an important cause of neonatal mortality.

The aim of the study was to investigate the metabolism of endotoxic neonatal rats and the potential beneficial effect of glutamine.

METHODS: Suckling rats received intraperitoneal saline (control; C), endotoxin (300 microg/g LPS; E), saline+glutamine (2 mmol/g; CG), endotoxin+glutamine (EG), saline+leucine (2 mmol/g; CL) or endotoxin+leucine (EL). Sepsis score (0-8) and rectal temperature were monitored. Hypothermia was defined as rectal temperature less than 32 degrees C. Oxygen consumption (VO2, mL/kg/h), a determinant of heat production, was measured by indirect calorimetry. Data (mean +/- SEM) were compared by analysis of variance (ANOVA), paired t test or Fisher's exact test.

RESULTS: Endotoxic (E) rats had significantly lower VO2 than C rats from 90 minutes post injection to the end of the experiment, 210 minutes (VO2 from 150 to 210 minutes: C 671 +/- 45; E 429 +/- 36, P <0.0004; n = 8; paired t test). VO2 of CL or CG rats was elevated between 90 and 210 minutes compared with control, but significantly (P <0.01) only in the L group (C 706 +/- 31; CG 871 +/- 63; CL 984 +/- 31; n = 7-9, ANOVA). VO2 was significantly higher (P <0.05) in EG rats than E rats (E 460 +/- 29; EG 654 +/- 68; n = 9-10). In the EL group, VO2 was raised but was not significantly different from E (E 460 +/- 29; EL 637 +/- 52; n = 8-10). EG rats were significantly less hypothermic between 90 and 210 minutes (58 of 132 measurements) compared with E (95 of 147; P =0.0007, Fisher's Exact test), whereas the EL group were similarly hypothermic (74 of 120) to E (P =0.7). Sepsis score was significantly lower in the EG group than both E

and EL groups (E 4.9 +/- 0.3; EG 3.6 +/- 0.3; EL 5.0 +/- 0.3; n = 40; P <0.01; ANOVA).

CONCLUSIONS: Neonatal endotoxaemia lowers VO2, heat production, and body temperature. Glutamine and leucine both cause nutrient-induced thermogenesis in control animals and restore VO2 of endotoxic animals. Glutamine additionally increases rectal temperature, reduces incidence of hypothermia, and improves clinical signs of endotoxic rats. This suggests that glutamine may be beneficial for nutrition in neonatal sepsis.

10.6.2 Joint ESPEN and BAPEN Conference Sep 2002 Glasgow, UK

BENEFICIAL EFFECTS OF GLUTAMINE IN NEONATAL ENDOTOXAEMIA

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Rationale: Sepsis is an important cause of neonatal mortality. The aim of the study was to investigate the metabolism of endotoxic neonatal rats and the potential beneficial effect of glutamine.

Method: 11d rats received intraperitoneal saline (control; C), endotoxin (300ug/g LPS) (E), saline+glutamine (2mmol/g; G), endotoxin+glutamine (EG), saline+leucine (2mmol/g; L) or endotoxin+leucine (EL). Hypothermia, defined as rectal temperature <32°C, and sepsis score (0-8) were monitored. Oxygen consumption (VO2, ml/kg/h), a determinant of heat production, was measured by indirect calorimetry. Data (mean +/- SEM) were compared by ANOVA, paired t-test or Fisher's exact test.

Results: Endotoxic (E) rats had significantly lower VO2 than C rats from 90min post-injection to the end of the experiment, 210min (E 512+/-58; C 776+/-30, $p<0.0001$ n=8 paired t-test). VO2 of L or G rats was consistently > than that of C group, but significantly so ($p<0.01$) only in the L group (C 706+/-31; G 870+/-63; L 984+/-32,n=7-9, ANOVA). VO2 of EG rats was significantly higher ($p<0.05$) than E whereas the EL group, although raised, was not significantly different from E (E 460+/-29; EG 654+/-68; EL 637+/-52 n=8-10). EG rats were significantly less hypothermic between 90-210 min (58/132 measurements) compared with E (95/147; p=0.007, Fisher's exact test) whereas the EL group were similarly hypothermic (74/120) to E (p=0.7). Sepsis score was significantly lower in the EG group than both E and EL groups (E 4.9+/-0.3; EG 3.6+/-0.3; EL 5.0+/-0.3; n=40 $p<0.01$ ANOVA).

Conclusions: Neonatal endotoxaemia lowers VO2, heat production and body temperature. Glutamine counteracts these effects, improves clinical signs, and may be beneficial for nutrition in neonatal sepsis.

10.6.3 BAPS Conference Estoril, Portugal, 2003

Glutamine decreases inflammation in neonatal sepsis

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Background: Neonatal sepsis increases systemic inflammation, which may lead to multiple organ failure and death. Glutamine has been suggested to have

beneficial effects during neonatal sepsis, but its effects on systemic inflammation are unknown.

Aim: To determine whether glutamine administration affects inflammation in neonatal endotoxaemia

Methods: 11 day old Wistar rat pups were given intraperitoneal injections (total volume 0.024ml/g in all groups) of saline (control; C), endotoxin (300μg/g lipopolysaccharide *E.Coli*) (E), saline with glutamine (2mmol/g; G), or endotoxin with glutamine (EG). Animals were sacrificed after 2, or 6 hours, exsanguinated and plasma separated. Plasma glutamine was measured enzymatically (expressed as mM, n=5 per group), and tumour necrosis factor alpha (TNF-alpha, expressed as pg/ml) levels measured by enzyme-linked immunosorbent assay (n=10 per group at 2h, n=7 per group at 6h). Results, expressed as mean ± SEM, were analysed by one-way ANOVA.

Results: Endotoxaemia caused a rapid significant decrease in plasma glutamine concentration at 2h (C 0.73±0.06; E 0.32±0.07 $p<0.001$), which was prevented by intraperitoneal glutamine (EG 0.59±0.04 $p<0.05$ *vs.* E), indicating absorption of glutamine. TNF-alpha levels were greatly increased by 2h endotoxaemia (C 27±7, E 2247±43 $p<0.001$) and this increase was partly prevented by glutamine (EG 1991±91, $p<0.01$ *vs.* E). The effect of glutamine was more pronounced at 6h (C 32±27, E 799±193, EG 219±75, $p<0.001$ C *vs.* E, $p<0.01$ E *vs.* EG).

Conclusions: Glutamine administration prevents the sepsis-induced fall in plasma glutamine levels, and reduces the concentration of a systemic inflammatory marker, supporting its use as a therapeutic agent in neonatal sepsis.

10.6.4 Submission to Clinical Sciences 2005

Glutamine decreases inflammation in suckling rat endotoxaemia

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Abstract:

Neonatal sepsis increases systemic inflammation, which may lead to multiple organ failure and death. Glutamine has been suggested to have beneficial effects during neonatal sepsis, but its effects on systemic inflammation are unknown. The aim of this study was to determine whether glutamine administration affects inflammation in neonatal endotoxaemia. 11d Wistar rat pups were given intraperitoneal injections of saline (control; C), endotoxin (300μg/g *E.Coli* lipopolysaccharide) (E), saline with glutamine (2mmol/g; G), or endotoxin with glutamine (EG). Animals were sacrificed after 2 or 6 hours. Plasma glutamine (mM) was measured enzymatically), and both TNF-α (pg/ml) and IL-10 measured by ELISA. Results, expressed as mean ± SEM, were analysed by one-way ANOVA. Endotoxaemia caused a rapid significant decrease in plasma glutamine concentration at 2h (C 0.73±0.06; E 0.32±0.07 $p<0.001$), which was prevented by intraperitoneal glutamine (EG 0.59±0.04 $p<0.05$ *vs.* E), indicating absorption of glutamine. TNF-α levels were greatly increased by 2h endotoxaemia (C 27±7, E 2247±43 $p<0.001$) and this increase was partly prevented by glutamine (EG 1991±91, $p<0.01$ *vs.* E). The effect of glutamine was more pronounced at 6h (C

32±27, E 799±193, EG 219±75, $p<0.001$ C *vs.* E, $p<0.01$ E *vs.* EG). IL-10 levels were also greatly increased by 2hr endotoxaemia (C=55±21, E=2429±58, EG=1989±177, $p<0.001$ C *vs.* E, $p<0.01$ E *vs.* EG). Glutamine administration prevents the sepsis-induced fall in plasma glutamine levels, and reduces the concentration of both pro- and anti- inflammatory cytokines.

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